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No. 1

A PROGRAM OF ANALYSIS AND SYNTHESIS

W. C. ALLEE

Managing Editor of *Physiological Zoölogy*

PROFESSOR C. M. CHILD'S timely article entitled "Lithium and Echinoderm Exogastrulation: With a Review of the Physiological Gradient Concept" inaugurates a series of synthesizing papers in *Physiological Zoölogy* which, it is hoped, will be both extended and useful. Biologists are fairly adequately served at present with publication outlets for detailed scientific analyses. There are also journals devoted to supplying bibliographic citations, abstracts, and reviews, or some combination of these, and a few which specialize in presenting syntheses of scientific material. To our knowledge no journal devoted primarily to publication of the results of biological research is attempting to combine synthesis with analysis and so aid in marking out conceptual trails through the mass of data that is accumulating with great rapidity. It is our purpose to make a beginning toward filling this need.

In earlier days, some traces of which happily remain, the lengthy discussions with which each major scientific paper concluded, attempted to place the detailed material just presented in relation to available knowledge and frequently to develop theoretical treatment for the whole. Now, with the growing trend toward shorter papers, the concluding section, still called a "Discussion," is frequently limited to a restatement in general terms of the main points of the paper (which are again given for the third time in more condensed form in the "Summary") and to a more or less hurried survey of other recent work in the immediate field. Most of us have written such papers and will probably continue to do so.

Under this system long series of clear-cut analytic papers dealing with a developing situation may appear from one or from a number of laboratories, with each paper presenting, under a comprehensive title, one idea, the supporting evidence neatly condensed, and one conclusion, all carefully prepared for ready reference. The title serves as an abstract of the whole, so that, even without more extended abstracting service, the essential contribution need not be overlooked. At times, long series of such detailed reports appear from the same laboratory with no indication of the general ideas, if any, which are being developed as a result of the research. Interested readers can only infer that the author and his associates are working toward some general synthesis.



It is expected that the present series will give a provocative opportunity for presenting three main types of synthesizing papers. In the first place, there will be a chance for a more comprehensive analysis of the many detailed analyses in the world's literature on a given subject; this constitutes one kind of synthesis. In turn, such competent surveys may lead to a synthesis of a higher order, including the development of suggestive theory. In a different category, which is illustrated by Professor Child's paper, we have those cases in which the development of theory has kept step with critical experimentation but in which there is need for a new evaluation of both facts and theory in the light of related developments. Finally, there are those fields in which all we can hope for at present is to have the whole developing situation reviewed by an expert. This will enable us to get the essential facts straight, so that investigation can proceed more intelligently. If skilfully done, these need not result in the obvious hash of the literature characteristic of too many review articles.

It is our conviction that detailed reports of investigations can best be evaluated and interpreted by one who has himself been actively engaged in research work in the field. Such a worker knows the technical pitfalls that may have weakened the supposed conclusions and the essential complexities that must be preserved. For our purposes, we distrust the work of the professional synthesizers. These belong to two or three schools. The first of these can readily be dismissed; their aim is to re-write scientific work so that it can be more readily understood by their public. They often serve a useful function in their own sphere, but our synthesizing series has an entirely different outreach.

Another type of professional synthesizer includes those who, on the basis of training in physical or in philosophical theory, undertake to interpret biological material which they understand imperfectly, in language which can be understood by few others. All too frequently the result is an oversimplification which cannot possibly fit the known facts or one which, despite the triumphant air of the synthesizer, has already been reached by laboratory workers. And while we appreciate verification of our results by others using radically different methods, there is doubtful value in the "discovery," even by a new method, of relations which are already well known.

There is no intention of decrying the aim of theoretical biophysicists whose work is now in its infancy. Perhaps the present attempts at biological synthesis by experimental biologists will furnish a solid and more complete basis for their efforts. Perhaps, too, the philosophers may find here dependable condensations which will be useful in their attempts to unify human knowledge. Unless modern philosophies are based on all phases of our knowledge, including that contributed by modern science, they are clearly inadequate. Equally obviously, the philosopher cannot be expected to master the original documents of science, and he should not be limited to the fields which, for one reason or another, are summarized in book form.

So much for the professional scholars; we have, also, a responsibility to the educated biological public and to students. These, too, deserve to be presented from time to time with readable summaries of the work in active fields of research made by competent research workers who are scholars as well as pioneers.

In order to make a start on this program, the University of Chicago Press has increased its subsidy to enable us to offer at least two synthesizing articles per volume without reducing the budgeted number of pages devoted to publishing the results of original research. The conduct of the analytical part of the journal will continue exactly as in the past, and our pages will remain open to all research papers within our field

which meet our standard of excellence. It may be appropriate at this point to emphasize that *Physiological Zoölogy* has no upper limit on the number of pages that will be published in one research article. The relative density of ideas per page and the editors' judgment of the value of the longer article, in comparison with the shorter ones which it replaces, are the criteria used in judging the longer manuscripts that are submitted.

In contrast to the open policy as regards research reports, the series of synthesizing papers will be entirely invitational. The responsibility of the editors will be limited to securing the contribution and to attempting to keep the article to a reasonable length, which we think, in general, should not exceed about fifty manuscript pages, bibliography included. With our present format this will run about twenty-five pages of print.¹

The following synthesizing articles are in preparation. The exact time and order of their appearance cannot be predicted. Others are being solicited and will be announced later.

E. E. Just, "Unsolved Problems in General Biology."

L. V. Heilbrunn, "The Relation of Protoplasm to the Calcium Ion."

B. H. Willier, "Embryonic Organization in Presomite Chick Blastoderms."

J. H. Bodine, Title to be announced—some phase of cellular physiology.

Alan Boyden, "Serology and Animal Relationship"

¹ A recent analysis gives the following type-load per page for these journals:

	Fms per Page
<i>Journal of Comparative and Cellular Physiology</i>	1,025
<i>Journal of Experimental Zoölogy</i>	1,025
<i>Biological Bulletin</i>	1,310
<i>Physiological Zoölogy</i>	2,010

LITHIUM AND ECHINODERM EXOGASTRULATION: WITH A REVIEW OF THE PHYSIOLOGICAL-GRADIENT CONCEPT

(Eighty-six figures)

C. M. CHILD

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IN EARLIER papers gradient patterns of normal sea-urchin and starfish development and their alterations in exogastrulae, as indicated by differential dye reduction in low oxygen, were described, and their relations to the primary pattern of echinoderm development were discussed (Child, 1936a, 1936b). The present paper is a further experimental study of exogastrulation and related modifications in the echinoid, *Dendraster excentricus*, with particular reference to their bearing on the general problem of developmental pattern. To this is appended a review of the physiological-gradient concept.

The experiments of Herbst made it evident that lithium is highly effective in producing exogastrulation, but it has also been shown that many other physical and chemical agents are also more or less effective. It is still maintained by some, however, that the action of lithium as an exogastrulating agent is in some way unique and indicative of regional localization or graded distribution of specific substances. For example, Runnström and his co-workers postulate two opposed and overlapping concentration gradients of specifically different substances—an “animal” and a “vegetative” (vegetal) gradient in the sea-urchin egg—and maintain that lithium favors, increases, and extends the

vegetal gradient, supposedly by favoring formation of the vegetal substance.¹ Apparently this is regarded as a specific chemical effect. It was pointed out in the paper cited above that it is difficult to interpret certain experimental data in terms of this hypothesis, for lithium does not always or necessarily increase entoderm at the expense of ectoderm but, under certain conditions, inhibits entoderm more than ectoderm. The method most frequently used to produce exogastrulation consists in exposure for a number of hours from early developmental stages to certain ranges of concentration of LiCl, the effective range differing with different species, followed by return to water. With this procedure exogastrulation may or may not occur before return to water, according to concentration of LiCl and exposure period. Certain ranges of concentration and exposure periods produce merely differential inhibition of early development, and exogastrulation occurs after return to water. With other concentrations and exposure periods exogastrulation may occur in the solution, but great changes in relative proportions of parts may follow on return to water, and development may proceed much farther than with continuous exposure to lithium. Also, other effects of continuous exposure may be very different from those of temporary exposure.

Interpretations of some of these effects have been suggested (Child, 1936b); but in general the question of their physiological significance has received comparatively little attention, and the possibility of secondary modifications resulting from differential tolerance, conditioning, or recovery has usually been ignored. Since this is the case, it may still be questioned whether or to what extent lithium exogastrulae of echinoderms depend on specific regional features of developmental pattern rather than on nonspecific regional differentials or gradients involving quantitative dynamic factors and differential susceptibility. It is believed that the experimental data presented in this paper throw some further light on this question.

To the director and the staff of the Hopkins Marine Laboratory I am again deeply indebted for their kindness in providing facilities and material, making these further experiments possible during the summer of 1938.

MATERIAL AND EXPERIMENTAL PROCEDURE

In the experiments of 1938 the sand dollar, *D. excentricus*, served exclusively as material; in earlier experiments *Strongylocentrotus purpuratus*, *S. franciscanus*, *Arbacia punctulata*, *Echinarachnius parma*, and the asteroids, *Patiria miniata* and *Asterias forbesii*, had been used. The 1938 experiments consisted primarily in a systematic study of effects of LiCl from the lowest concentrations producing any appreciable modification of development to those completely inhibitory and lethal, that is, fifteen concentrations from m/120 to m/10. Most of these concentrations were used both in approximately isotonic and in somewhat hypertonic sea-water solutions, but the only difference observed was that in the higher concentrations the hypertonic solutions were somewhat more effective in producing exogastrulation and other modifications. All concentrations given below are approximately isotonic unless otherwise stated. In addition to the wide range of concentrations, a wide range of exposure periods was also used: from 1-2 hours to continuous from first cleavage; and exposure was begun at different stages: first cleavage, sixteen to thirty-two cells, early, middle, and late blastulae, mesenchyme immigration, beginning and mid-gastrulation. Experimental lots, some one hundred and

¹ See Lindahl, 1936, and Runnström's papers cited by him.

fifty in number, were examined at frequent intervals in order to determine the course of developmental modification as well as the forms finally attained. These experiments, performed at the height of the *Dendraster* breeding season, showed a somewhat lower susceptibility of corresponding stages to LiCl than those of 1935, which were on material at the extreme end of the breeding season, when ripe gonads were still present in less than 1 per cent of animals collected. With that material greater or less degrees of exogastrulation occurred rather frequently in water, but only very rarely in a few individuals in the material of 1938. In lithium experiments of 1935 with *Patiria* as material, m/10, m/20, m/25, and m/30 were used on many lots with exposure beginning at different stages and for different periods. Earlier experiments with *Arbacia*, *Echinarachnius*, and *Asterias* were chiefly concerned with a few concentrations found to produce exogastrulation; but the results obtained, though fragmentary, were essentially similar to those of recent work as regards developmental modifications. Some data on exogastrulation by other agents have also been obtained.

Except in a few experiments on crowding, development took place in approximately 250 cc. of solution or water in practically flat-bottomed finger bowls set in flowing sea water and glass-covered in diffuse daylight. Care was taken to avoid crowding, and aggregations were often dispersed by gentle stirring. Approximate percentages of exogastrulae were noted with most lots, also high or low frequency of other modifications; but the chief purpose of the experiments was the study of the characteristic features of developmental modifications of individuals rather than their frequencies.

Figures are semidiagrammatic but based on ocular micrometer measurements of the various dimensions of living individuals, immobilized if necessary. The measurements were supplemented by many hundred sketches. General distribution of mesenchyme and of cells given off from the entoderm is approximately indicated in diagrammatic manner. Position and general pattern of skeleton is shown essentially as recorded in the sketches. Skeletons of anal arms are represented as single rods, though usually consisting of two apposed rods with cross-connections and in modified forms sometimes of three or four rods. Pigment is not indicated.

DIFFERENTIAL AND SPECIFIC SUSCEPTIBILITIES TO EXTERNAL AGENTS

The question whether echinoderm exogastrulation represents a specific regional action of external agents was discussed earlier (Child, 1936b). It was pointed out that, since exogastrulation is produced by many agents,² it cannot be regarded as a specific effect of any particular agent. Some agents are more effective than others, and lithium appears to be particularly effective. The high effectiveness of lithium is doubtless associated in some manner with its point of attack on living protoplasm, but this does not necessarily mean that its effect is on a regionally localized specific substance or substance-complex. The question of differential susceptibility of organisms to external agents has been repeatedly discussed,³ but it seems necessary to call attention again to certain points as a basis for presentation of experimental data.

² It is not necessary to repeat the list of agents given in the earlier paper, but nickel chloride is to be added (Waterman, 1937); and the occasional occurrence of a few exogastrulae with di-nitro-compounds, iodoacetic acid, and pyocyanine is reported (Waterman, 1938).

³ Child, 1928, and references given there to earlier work on differential susceptibility. In the earlier paper on exogastrulation it was briefly discussed with particular reference to echinoderm development (Child, 1936b, pp. 458-61).

It has often been pointed out that susceptibilities of early developmental stages of various animals and of the adults of many of the simpler organisms show a gradation or differential in the same direction in relation to axiate pattern with many chemical and physical agents and conditions in certain ranges of concentration or intensity. This differential susceptibility does not give evidence of specific substances regionally localized along a physiological axis with corresponding specifically different regional effects of different external agents. It suggests, rather, that axiate pattern is primarily a quantitative gradation or gradient in physiological condition. If this is granted, it appears beyond question that regionally localized specific differentiations, chemical and morphological, originate within this gradient pattern; when a certain degree of such differentiation is attained, specific differences in susceptibility begin to appear, and with further progress of differentiation become more distinct.

Differential susceptibility appears in various aspects. In direct differential lethal action and in differential inhibition of development a certain parallelism appears between susceptibility and rate of physiological activity, as indicated by rate of respiration, rate of dye reduction in low oxygen, and in many cases by rate of developmental activities. Regions most intensely active are most susceptible to direct lethal and inhibitory action, and the susceptibility gradient is, in general, parallel to the physiological gradient. With certain lower ranges of concentration or intensity of external agents differential tolerance, differential acclimation or conditioning, and, after temporary exposure, differential recovery may occur as secondary effects. In these secondary effects the regions most susceptible to the higher, directly lethal, or inhibitory ranges of concentration or intensity show, in general, the greatest tolerance, become acclimated or conditioned with continued exposure to the agent, or recover after temporary exposure most rapidly or completely. Tolerance, conditioning, and recovery decrease along an axis in the same direction as physiological activity. With certain ranges of action of agents, partial conditioning or recovery may occur, that is, a part of the organism may be killed or so inhibited that secondary changes are impossible while a less susceptible part may live long enough to become more or less conditioned or may recover on removal from the agent. These partial secondary effects are, as might be expected, most conspicuous in recovery. More susceptible regions may be killed, and the rest of the organism may recover after removal from the agent.

The susceptibility gradients are not directly related to the particular ways in which different agents interfere with the protoplasmic system. It appears evident that any sort of interference sufficient in degree will be more rapidly or more intensely effective in regions of greater, than in those of less, physiological activity, and that the more active regions will, in general, show greater tolerance or become conditioned to, or recover more rapidly or completely from, slight interference than the less active regions. Perhaps tolerance, conditioning, and recovery are not possible with some agents; but it is certain that they do occur with many.

These various expressions of differential susceptibility make possible differential modification of development in two opposite directions. In differential inhibition the "high" regions of the physiological gradients are most inhibited, most reduced in size, or may even be killed. In differential tolerance, conditioning, and recovery the high regions of the gradients, though primarily most inhibited, show secondarily the greatest development.

The absence of regional specific susceptibilities along a physiological axis does not

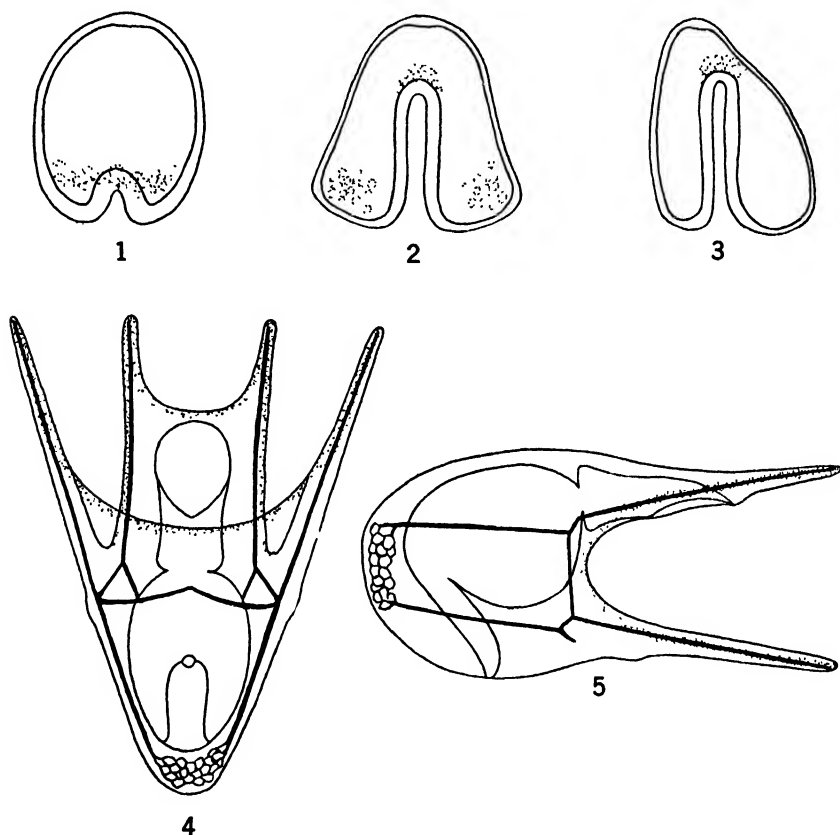
necessarily mean that qualitative or specific differences of substance are absent in that axis. At most, it can mean only that such differences are not sufficient to become a basis for specific differences in susceptibility. In as complex a system as a living protoplasm, the many differences in concentration of a multitude of substances and in condition of the colloid substrate, associated with or resulting from the differences in rate of metabolism at different levels of a gradient originating as a gradient in rate, provide ample basis for the origin of regional qualitative differences. These may begin to appear from the moment of establishment of a quantitative physiological gradient, but quantitative factors may remain predominant for a shorter or longer time. Probably so-called "determination" of a part is associated with attainment of a certain degree of specific or qualitative character by that part of a gradient pattern. The following experimental data bear upon the question whether or to what extent such localizations are primarily concerned in exogastrulation and related modifications of early development.

LARVAL DEVELOPMENT OF *Dendraster* UNDER NATURAL CONDITIONS

The early gastrula, the later gastrula, viewed laterally and ventrally, and the pluteus in anal and lateral view are shown in Figures 1-5. It will be noted in Figure 3 that the ventral side of the advanced gastrula and prepluteus becomes almost flat and that the tip of the archenteron approaches the dorsal wall before it bends ventrally toward the stomodeal region. The oral lobe of the pluteus develops from the apical region ("animal" region), as in other echinoids. It is proportionally considerably larger than the oral lobes of *Arbacia* and *Strongylocentrotus*. The short oral arms are parallel or almost parallel, and the angle between the anal arms (brachial angle) does not vary greatly. These figures serve as a basis for comparison with the alterations of form and proportions under experimental conditions.

Differential reduction of dyes in low oxygen (Child, 1936a) and differential death in various agents indicate that the apical region is in early stages the high end; the basal ("vegetal") region, the low end of the primary apicobasal gradient; and that, as gastrulation approaches, the basal region—mesenchyme and entoderm—undergo a change in condition, apparently a rather intense activation of some kind, indicated by marked increase in rate of dye reduction and in lethal susceptibility. Development of the oral lobe from the apical region apparently involves a secondary activation there in later gastrula stages, and each developing anal arm is a region of increased rate of dye reduction and increased susceptibility. In earlier blastula stages no difference in rate of dye reduction or susceptibility, indicating ventrodorsality, has been observed. In the more advanced blastula, still radial in form, rate of dye reduction and susceptibility become distinctly higher on one side. That this is the ventral side, distinguishable physiologically before it is morphologically, is highly probable from the fact that the ventral side, as soon as directly distinguishable, reduces dye more rapidly and is more susceptible than the dorsal. According to these observations, the ventral side appears at first to be merely the higher levels of a ventrodorsal gradient; but later the stomodeal region and anal arm primordia become distinguishable as regions of higher rate of dye reduction than the rest of the ventral side, the rate decreasing from a center in each. The gradual appearance of the ventrodorsal gradient in the course of pregastrular development, as indicated by the methods employed, is a point of some interest in relation to experimental modifications. For example, with differential inhibition beginning in early stages, ventrodorsality may never develop at all; but inhibition beginning in later blastula or in gastrula stages

usually merely decreases its developmental expression. During and immediately after immigration of mesenchyme, the rate of dye reduction becomes more rapid in the mesenchyme than in any other part, and more rapid in entoderm than in ectoderm; also, the invaginating entoderm develops a dye reduction gradient with high end at the tip, and experiments indicate a parallel differential susceptibility. In the fully developed pluteus the various developmental gradients disappear or become less distinct, but nothing is known concerning origin of new gradient systems in metamorphosis.



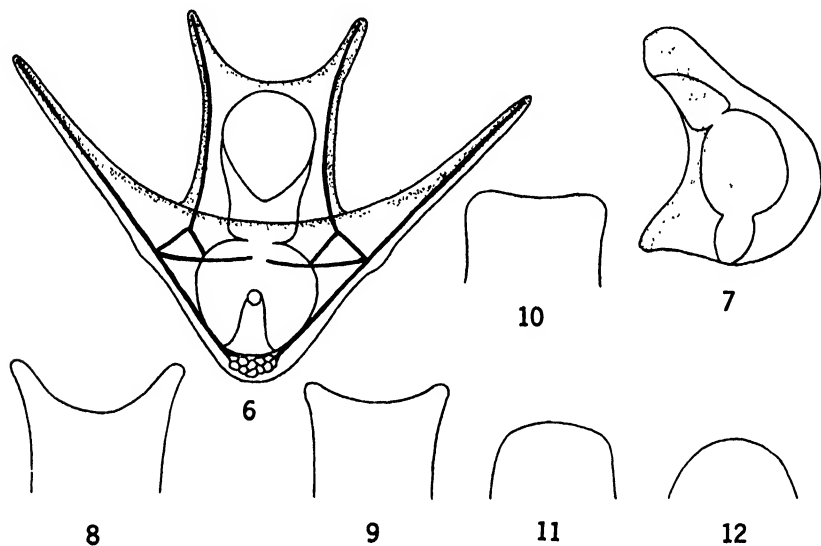
FIGS. 1-5—Larval development of *D. excentricus* under natural conditions. Fig. 1, early gastrula; Figs. 2 and 3, ventral and lateral outlines of later gastrula; Figs. 4 and 5, pluteus in anal and lateral view.

LITHIUM MODIFICATIONS WITH DIFFERENT EXPOSURE PERIODS FROM EARLY CLEAVAGE

Concentrations of LiCl below m/90 or m/80 rarely produce exogastrulae but do modify development differentially. The forms produced are of interest in comparison with certain characteristics of exogastrulae and require brief notice. Continuous exposure from first or early cleavage to m/120 produces characteristically plutei of the type of Figure 6 with large oral lobe, diverging oral arms, brachial angle wider than normal, and large mouth and foregut (cf. Figs. 4 and 6). Development is primarily retarded, as com-

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pared with controls; and the pluteus form is a secondary modification, resulting from greater tolerance or conditioning to lithium of the higher levels of the apicobasal and ventrodorsal gradients and probably also of the high end of the archenteric gradient. Development in $m/110$ and $m/100$ usually produces forms of the same type, except that oral arms are shorter or absent and that the oral lobe is merely rounded in many individuals; that is, more inhibition and less secondary modification of this region occurs. Anal arms are usually also somewhat shorter, and the angle less wide. All these forms are essentially similar to the wide-angled plutei of *Arbacia* developing in differential tolerance, conditioning, and recovery with various agents, though less extreme than



FIGS. 6-12.—Development with continuous exposure from first cleavage to the lowest differentially effective concentrations of LiCl. Fig. 6, differential tolerance or conditioning after 66 hours in $m/120$, indicated by enlarged oral lobe, wide angle of oral and anal arms, and probably by large esophagus; Fig. 7, the most inhibited after 39 hours in $m/90$ with slight secondary modification apically; Figs. 8-12, outlines of oral lobes in $m/90$, ranging from wide-angled with more or less inhibited oral arms (Figs. 8 and 9) to differentially inhibited lobes (Figs. 10-12).

some of those produced as secondary modifications with agents which permit early tolerance, conditioning, and recovery.

In $m/90$ continuously from first cleavage the plutei range from somewhat wide-angled forms with large oral lobe, but with oral arms more or less inhibited and anal arms shorter than normal, to forms with only slight secondary modification of the apical region and very short or no anal arms (Fig. 7). Figures 8-12 show outlines of oral lobes of these forms, ranging from wide angled, but with oral arms somewhat inhibited (Figs. 8 and 9), through various degrees of differential inhibition (Figs. 10-12).

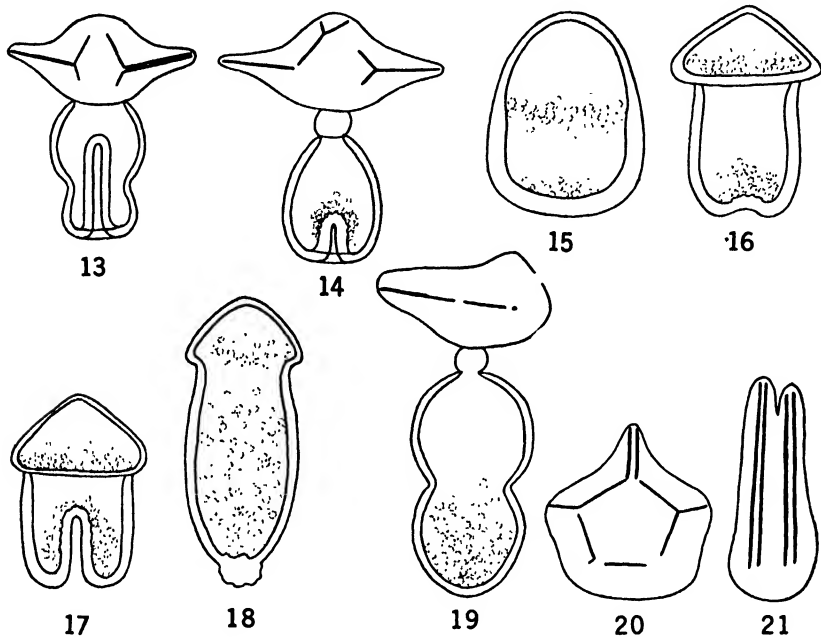
With continuous exposure to $m/80$ there is no certain evidence of secondary modification. The plutei are smaller than controls; the oral lobe is differentially inhibited and without oral arms; the anal arms are only about half the control length; and the relative size of entodermal divisions differs little from normal. Animals returned from all these

low concentrations to water a few hours before gastrulation develop a high percentage of plutei like controls; but a few slightly wide-angled forms with large oral lobe may appear, indicating slight degrees of differential recovery.

EXOGASTRULATION AND ENTODERMIZATION IN THE LOWER EFFECTIVE CONCENTRATIONS

With sufficiently long or continuous exposure from first or second cleavage to $m/70$ or $m/60$ the forms commonly known as exogastrulae appear in percentages ranging from 10 to 50 in different lots. Most of these, however, are entexogastrulae; examinations during their development show that the original entoderm is completely or partially invaginated—but is inhibited in further development, and additional entoderm, which shows no tendency to invaginate, originates from entodermization of prospective ectoderm by lithium. With these concentrations entodermization involves only the more basal levels of prospective ectoderm (Figs. 13 and 14). Certain features of these forms require attention. First, lithium not only transforms more or less of the prospective ectoderm into entoderm but also inhibits development of the original prospective entoderm. The invaginated entoderm shows no differentiation into the usual three regions, ends free in the blastocoel, and in Figure 14 is giving off cells additional to the primary mesenchyme, an effect of inhibiting conditions, as will be more clearly evident below. Second, the entodermized ectoderm which forms most or all of the external entoderm of these forms remains in essentially the same relation to the rest of the individual as when it was prospective ectoderm. It has become entoderm but has not evaginated, instead of invaginating; and it has not changed its position. Are, then, forms like Figures 13 and 14, strictly speaking, exogastrulae? This question arises as regards many other so-called exogastrulae and will be discussed later. Meanwhile the terms "exogastrulation" and "exogastrula" will serve for descriptive purposes as indicating the condition or individual with noninvaginated entoderm, but without implication as to origin of the entoderm. Exogastrulation of the original prospective entoderm does occur in many individuals of the same lots as Figures 13 and 14; it may be partly evaginated in Figure 14. However, as will appear, there are many forms with noninvaginated entoderm in which the original prospective entoderm is only in small part or not at all involved. Third, development of the entodermized ectoderm is inhibited in lithium; with continuous exposure it does not develop far beyond the condition shown in Figures 13 and 14. Fourth, the apical region of the remaining ectoderm in these forms is greatly inhibited, but anal arms develop at a wide angle. Conditions determining these modifications, however, are quite different from those which result in wide-angled plutei like Figure 6. It was noted above that the ventral side of the advanced gastrula is flat (Fig. 3). In forms like Figures 13 and 14 and in other similar forms described and figured below, further development of the ventral side is inhibited to such a degree that it remains almost flat or may, under certain conditions, become convex instead of deeply concave, as in normal plutei (Figs. 1 and 2). Moreover, the ectoderm of Figures 13 and 14 is only the apical half more or less of the prospective entoderm. Development of this region involves extensive reconstitution and decrease in scale of organization. Arms develop from ectoderm which, under natural conditions, would give rise to parts intermediate between arms and oral lobe or to proximal parts of the lobe. Arm development is localized at the lateral borders of the ventral side of the region retaining ectodermal character; but since this remains more or less flattened or becomes convex, arms grow out at a wide angle, conditions on the ven-

tral side apparently being the chief factors in determining direction of their growth, perhaps by determining direction of growth of skeletal rods. According to this interpretation, forms of this general type represent primarily differential inhibitions. Development of prospective entoderm, entodermized ectoderm, and remaining ectoderm is inhibited; in the remaining ectoderm apical and ventral regions are most inhibited. However, activation and outgrowth of arm tissues, a feature of relatively late stages, and perhaps also some relative increase of ventral area, may represent some degree of dif-



FIGS. 13-21.—Modifications by $m/70$, $m/60$, and $m/50$. Figs. 13 and 14, modifications occurring in percentages from 10 to 50 with continuous exposure from first cleavage to $m/70$ or $m/60$. Fig. 15, 15-18 hours in $m/50$ from two-cell stage, entodermization of basal prospective ectoderm, dissociation of cells from original prospective entoderm; Figs. 16-18, most advanced stages attained in $m/50$; Figs. 19-21, development following return to water from $m/50$ at stage of Fig. 15; Fig. 20, ectoderm of Fig. 19; Fig. 21, another ectoderm with fused arms.

ferential conditioning in the cases of Figures 13 and 14 or of recovery in various other similar forms (see Figs. 32, 33, and 46); but development of the oral lobe from the apical region, primarily the most susceptible region of the embryo, is completely inhibited. It is also possible that outgrowth of arms is determined by the skeletal rods. In any case, secondary modification in these forms is slight, if present at all; they differ greatly from the secondary modifications after slight inhibition, such as Figure 6.

With continuous exposure to $m/70$ or $m/60$, beginning with the 5-hour blastula, plutei with slightly inhibited oral lobe and anal arms result. These concentrations are not high enough to produce exogastrulation or entodermization from these stages on, but higher concentrations are still effective at this stage. Evidently "determination" of parts

has become somewhat more stable during 5 hours of development. With return to water from m/70 or m/60 before development is too far advanced, some evidence of differential recovery appears, but higher concentrations provide more interesting examples.

THE HIGHER CONCENTRATIONS

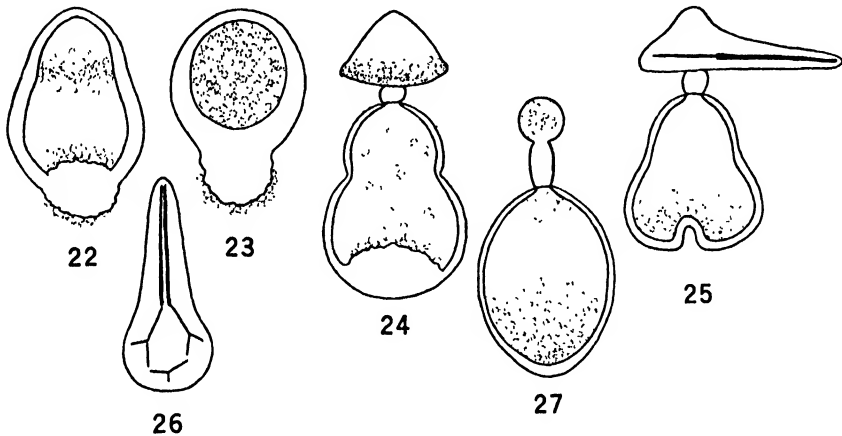
Concentrations of m/50 and above are highly effective in inhibiting development in *Dendraster*; and with certain exposure periods at early stages, effective in producing exogastrulation. The usual condition after 15–18 hours in m/50 from two-cell stage is shown in Figure 15. The circle of primary mesenchyme, which immigrates to a level slightly apical to the ecto-entodermal boundary, is in these forms about midway between the poles; that is, the basal half, more or less, of the prospective ectoderm has become entoderm. In a few individuals some invagination of prospective entoderm has taken place, but in all there is dissociation of cells from it. The most advanced stages attained with continuous exposure are shown in Figures 16–18. The ectoderm remains radial; the primary mesenchyme remains, in large part, in a circle and does not form skeleton; in many individuals the prospective entoderm continues to give off cells into the blastocoel or may lose more or less completely its epithelial character and become a solid cell mass (Fig. 18). Evidently its further development is inhibited, even when it invaginates (Fig. 17). Following return to water at the stage of Figure 15 the entodermized ectoderm elongates and usually differentiates into two regions (Fig. 19). The small “neck” connecting ectoderm and entoderm appears very commonly in exogastrulae as a secondary modification; its possible significance is discussed later. The remaining ectoderm recovers sufficiently to develop a differentially inhibited ventrodorsality with a single arm (Figs. 19 and 20), fused arms (Fig. 21), arms diverging at sub-normal angle, and a few wide-angled individuals with inhibited oral lobes. Further development of entodermized ectoderm also results from recovery, but the original entoderm gives no definite evidence of recovery and may be largely dissociated into free cells in the blastocoel.

Figure 22 shows the characteristic condition after 18 hours in m/35; extremes under these conditions are like Figures 15 and 23. In Figure 22 entodermization extends farther apically than in Figure 15, and the original entoderm has lost its epithelial order and is undergoing dissociation. In Figure 23 most or all of the original entoderm is a solid mass of cells dissociating externally but has probably also given off cells into the blastocoel. Development does not proceed much farther in this concentration. Some enlargement and decrease in thickness of the cell wall may occur; the original entoderm apparently undergoes complete dissociation; sooner or later the entodermized ectoderm begins to dissociate; and the form of the whole gradually becomes spherical and remains so until death.

In lots returned to water at the stage of Figures 22 and 23 the characteristic form is of the type of Figure 24, with a radial ectoderm, a circular transverse ciliated band about its basal region, the original entoderm a more or less dissociated cell mass without pattern, and entodermized ectoderm large, with two regions separated by a constriction. At one extreme a few individuals develop a one-armed ectoderm, and one case of partial invagination was recorded (Figs. 25 and 26). At the other extreme are almost completely entodermized forms with original entoderm largely or wholly dissociated and with little or no enlargement of entodermized ectoderm, that is, with little or no evidence of recovery (Fig. 27).

With certain exposure periods $m/30$ and $m/25$ produce 100 per cent exogastrulae. A number of series were run in these concentrations with continuous exposure and with return to water after different exposure periods of portions of the same lot. After 23 hours in $m/30$ from first cleavage the original entoderm is a cell mass undergoing dissociation either in the blastocoel or outside, and prospective ectoderm is in large part or almost wholly entodermized (Figs. 28 and 29). The condition of those still alive after 47 hours in $m/30$ is shown in Figures 30 and 31. Approach to spherical form, dissociation of cells from the remaining epithelial wall, and death result from longer exposure.

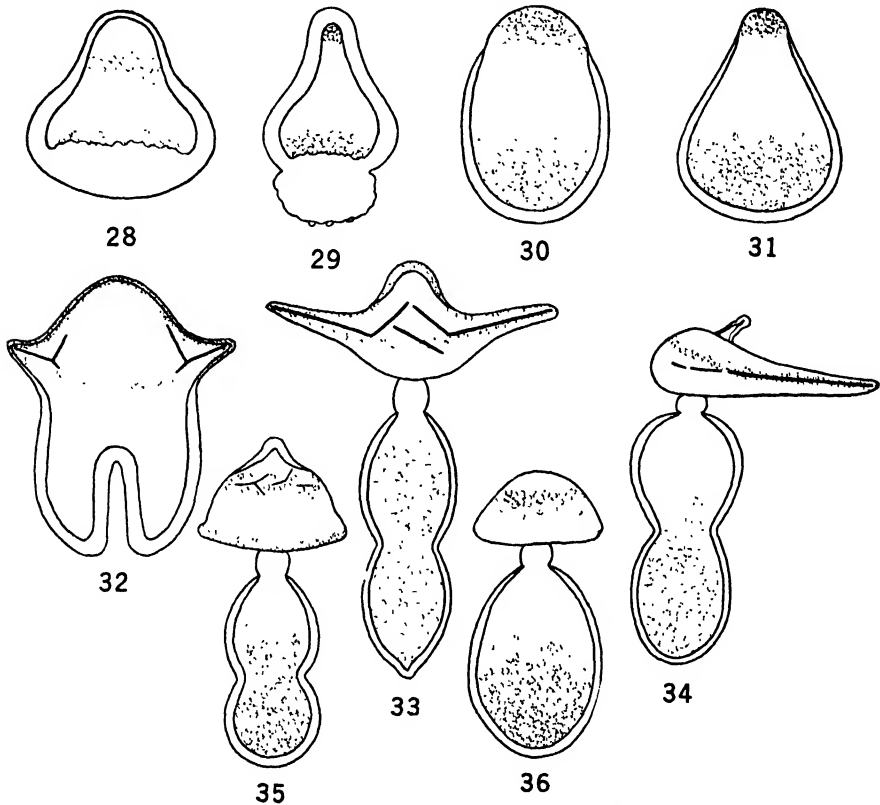
Animals from the same lot returned to water after 3 hours become entogastrulae and develop into normal plutei. In water after 5 hours' exposure the original prospective entoderm invaginates, entodermization of about the basal half of the prospective ecto-



FIGS. 22-27.—Effects of $m/35$ LiCl. Figs. 22 and 23, 18 hours in $m/35$ from first cleavage; Fig. 24, the characteristic form developing following return to water after 18 hours' exposure; Figs. 25-27, extreme forms of same lot.

derm occurs, entexogastrulae result, and the remaining ectoderm develops ventrodorsality (Fig. 32) and finally approaches pluteus form with inhibited oral lobe and short arms at varying wide angles. In a few individuals a single apical coelom, apparently without bilaterality, develops from the invaginated entoderm, as in Figure 45, evidently a differential inhibition of this region; but no further regional differentiation of entoderm appears. In water after $8\frac{1}{2}$ hours' exposure, there is no invagination of entoderm (Figs. 33-35). Fifteen hours after the return of this lot to water the original entoderm was wholly or almost wholly dissociated. Some of its cells perhaps form a part of the terminal entodermal region of these forms, but most of them are free in the blastocoel or lost externally, and the entodermal epithelium is largely or wholly entodermized ectoderm. Its large size (Figs. 33-35) results not from a direct effect of lithium but from differential recovery after return to water. Ectodermal development ranges from forms with short, wide-angled arms through one-armed forms to completely radial. The secondary thickening of the apical region in some individuals (Figs. 33 and 35) indicates slight differential recovery. Mesenchyme in the radial forms lies near the apical region, and a circle of spicules often develops there (Fig. 35). Since the circle of mesenchyme is near the ecto-

entodermal boundary in earlier stages (Figs. 15, 22, 28, and 29), its position in these forms suggests that there may have been a re-ectodermization of entodermized ectoderm after return to water. Occasionally an evaginated, relatively large stomodeum develops with indication of two more or less distinct regions (Fig. 34). The evagination may be a lithium effect, but the large size is undoubtedly a result of differential recovery. In water



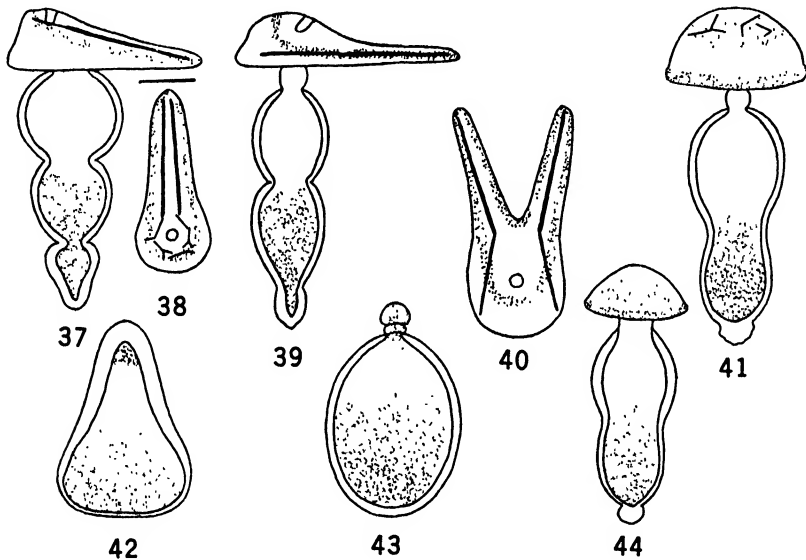
FIGS. 28-36.— Modifications of single lot of eggs with different exposure periods to $m/30$ from first cleavage. Figs. 28 and 29, continuous, 23 hours; Figs. 30 and 31, continuous, 47 hours; Fig. 32, 22 hours in water after 5 hours in $LiCl$, further approach of ectoderm to pluteus form in later stages, not figured; Figs. 33-35, forms developing in water after $8\frac{1}{2}$ hours in $m/30$, with evidence of differential recovery of apical region in Figs. 33 and 35, of entodermized ectoderm in all, and in development of everted stomodeum in Fig. 34; Fig. 36, development in water after 10 hours in $m/30$.

after 10 hours' exposure to $m/30$ there is less recovery, less enlargement of entodermized ectoderm, and the remaining ectoderm is always radial with circle of mesenchyme or a few spicules near its apical pole, again suggesting some re-ectodermization (Fig. 36). When a ciliated band develops on these ectoderms, it is a transverse circle at the margin of the flat basal region of the ectoderm.

A few forms from other lots of eggs in $m/30$ are shown in Figures 37-44. Figures 37-41 are forms developing in water after $18\frac{1}{2}$ hours' exposure from the eight- to the

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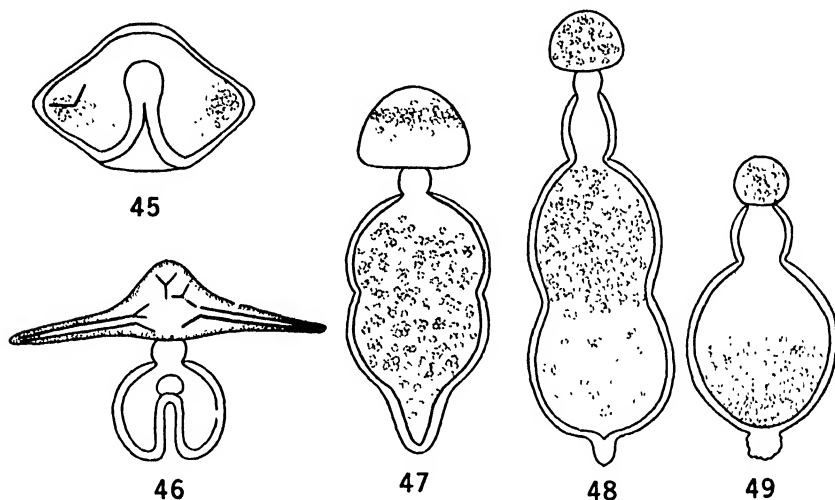
sixteen-cell stage. They show somewhat more development of entodermized ectoderm than Figures 35 and 36, although longer in LiCl. In Figures 37, 39, and 41 the terminal region of the entoderm is probably a part of the original prospective entoderm. In Figure 41 the skeletal spicules are in the apical region; in many individuals of the lot spicules or mesenchyme cells are in this position, indicating probable re-ectodermization after return to water. Figures 42-44 from another lot show the condition after 27 hours in m/30 from first cleavage (Fig. 42) and the range of forms developing after return to water at this time (Figs. 43 and 44). The thin epithelium in the basal region of Figure 42 seems to be part of the original entoderm which has not dissociated. This condition appears frequently.



FIGS. 37-44.—Other examples of modification with different exposure periods to m/30. Figs. 37 and 39, lateral, and Figs. 38 and 40, apical, views of forms developing in water after 18½ hours in m/30 from the eight- to the sixteen-cell stage; Fig. 41, radial form from same lot; Figs. 42-44, return to water after 27 hours in m/30 (Fig. 42) and range of forms developing later (Figs. 43 and 44).

Series with different exposure periods to m/25 from first cleavage present some further points of interest. With development in water after 3 hours' exposure, entoderm invaginates in all, and only about 10 per cent show any entodermization of prospective ectoderm; but after 22 hours in water, differential inhibition is still evident: the apical ectodermal region is inhibited, oral lobe is absent, and the apical entoderm is often a solid cell mass (Fig. 45); but these forms gradually undergo more or less differential recovery and may become small plutei with inhibited oral lobe and short arms. In water after 5 hours' exposure invagination of entoderm often occurs, but there is usually more or less dissociation, with or without invagination. In Figure 46 the most highly developed individual observed in the lot, an apical coelom with no evidence of bilaterality, develops from the invaginated entoderm. Like the similar case mentioned above, this results from a mediolateral differential inhibition essentially like that producing approxi-

mated, fused, and single anal arms in echinoid development and approximation of bilateral cephalic organs and cyclopia in planarians and in vertebrates. The characteristic form of this lot is Figure 47 with radial ectoderm, subapical circle of mesenchyme, large entodermized ectoderm, and more or less complete dissociation of original entoderm. In water after $7\frac{1}{2}$ hours' exposure, all individuals become essentially like Figure 47, except that the ectoderm averages somewhat smaller. In all these the mesenchyme is subapical, as in Figure 47, probably in consequence of re-ectodermization during recovery. The characteristic condition after $21\frac{1}{2}$ hours' exposure is very similar to that shown in Figure 42, except that the original entoderm is still largely a solid mass. Figures 48 and 49 show the most advanced development after return to water at this time: in these the cell mass at the tip of the entodermized ectoderm probably represents part of the original entoderm.

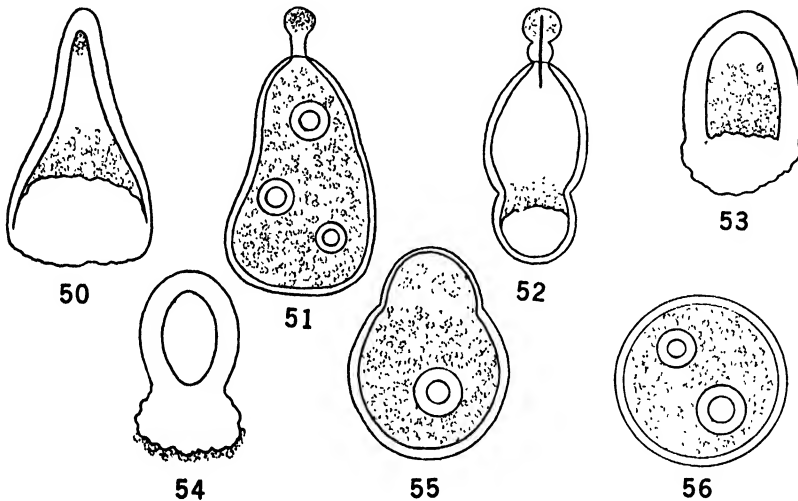


FIGS. 45-49.—Modifications by $m/25$ with various exposure periods from first cleavage. Fig. 45, 3 hours in $m/25$, 22 hours in water, differential inhibition decreasing basipetally in ectoderm and in archenteron; Fig. 46, most highly developed larva in water after 5 hours in $m/25$; Fig. 47, the characteristic form developing in water after 5 hours in $m/25$; Figs. 48 and 49, characteristic development in water after $21\frac{1}{2}$ hours in $m/25$.

It is a point of some importance that with all the shorter periods of exposure to $m/30$ and $m/25$ (less than 10 hours) the animals at the time of return to water are, in any case, only slightly different from the controls. Even with the longer of these exposures there is only a slight differential inhibition decreasing from the apical region basipetally indicated by smaller size of the apical region than in controls and retardation of the decrease in thickness of the blastula wall. All further modifications of development occur after return to water and evidently represent combinations of the direct inhibiting effect of lithium and the secondary modifications associated with recovery from that effect. The lithium effect is progressive, and recovery from it is less rapid and less complete the longer the exposure from any given stage of development.

Even with $m/20$ certain results of interest appear. In approximately isotonic $m/20$ only large-celled early blastula stages are attained after 9 hours; controls are advanced

blastulae. With return to water at this time, characteristic forms 17 hours later are like Figures 42 and 50. The whole basal region has lost epithelial character and is more or less completely dissociated; in some individuals pigment appears in the extreme apical region. These forms stick together in large numbers by their basal regions, with the more apical epithelial portions protruding from the surface of the mass, and gradually become large, irregular masses full of dissociated cells; dissociation may finally become complete except for an external epithelium surrounding the multiple mass. All the individuals involved in one of these masses have lost all individuality, and most of their cells have become free cells in the interior of the mass; only a surface-interior pattern remains, and death finally occurs without further change. Forms like Figure 50, if they

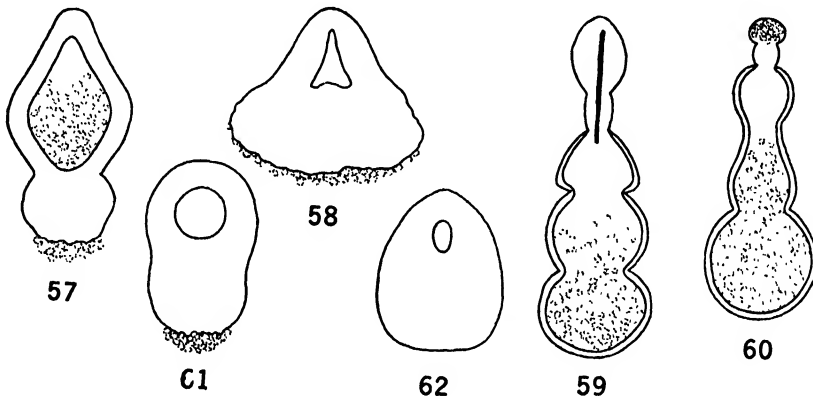


FIGS. 50-56.—Modifications with different exposure periods from the first cleavage to $m/20$. Fig. 50, 9 hours in $m/20$, 17 hours in water; Figs. 51 and 52, 9 hours in $m/20$, 40 hours in water, entodermal epithelial vesicles in Fig. 51, a single skeletal rod in Fig. 52. Figs. 53 and 54, 26 hours in $m/20$; Figs. 55 and 56, 26 hours in $m/20$, 24 hours in water, apparently some re-ectodermization in Fig. 55, with Fig. 56 probably all entoderm.

remain single, may develop, as shown in Figures 51 and 52. In many of them one or more spherical epithelial vesicles form free in the blastocoel from some of the entoderm cells (Fig. 51). Similar epithelial vesicles often appear in considerable numbers in the large multiple masses if they are returned to water early enough. Single individuals occasionally develop a single skeletal rod extending apicobasally (Fig. 52). After 26 hours in $m/20$ entodermization of ectoderm is apparently complete; and dissociation, either into the blastocoel or externally or both, is progressing acropetally from the basal pole (Figs. 53 and 54). In some individuals cytolysis is beginning in the apical region. Many are still within the fertilization membrane. After return to water at this stage only 20-25 per cent remain alive for 24 hours, but these show some evidence of recovery. In some of them more or less ectoderm develops (Fig. 55), although the earlier stages show no visible evidence of any remaining ectoderm (Figs. 53 and 54). Re-ectodermization seems to have occurred in these cases, as in so many others. Other individuals are

spherical, apparently completely anaxiate with thin surface epithelium probably consisting entirely of entodermized ectoderm (Fig. 56), though it is not possible to determine whether it is to be regarded as entoderm or as re-ectodermized. One to several epithelial vesicles are present in almost all (Figs. 55 and 56), and many of these vesicles from disintegrated individuals persist free in the water. The reappearance of epithelial character in entodermal cells is evidently a result of recovery. Whether it represents cell aggregation or results from cell division has not been determined.

A sea-water solution of LiCl $m/20$ is, of course, highly hypertonic, and its effects are somewhat more extreme in certain respects than those of approximately isotonic solution. The condition after 7 hours in this solution from first cleavage and 12 hours in water is shown in Figures 57 and 58. Formation of a solid cell mass progressing acropetally from the basal region, usually with more or less dissociation of cells from its surface,



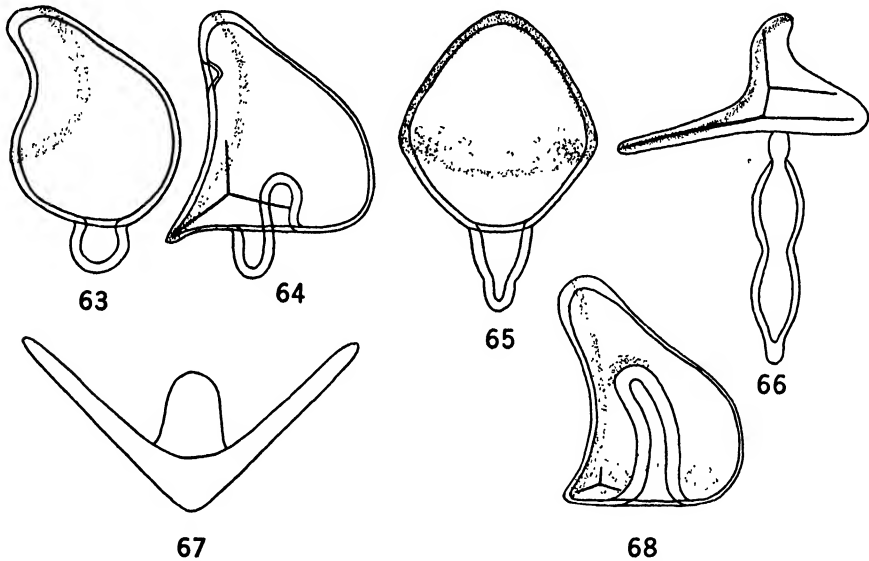
FIGS. 57-62.—Exposure from first cleavage to $m/20$ in sea water (hypertonic). Figs. 57 and 58, 7 hours in $m/20$, 12 hours in water; Figs. 59 and 60, 7 hours in $m/20$, 37 hours in water; Figs. 61, 62, 19 hours in $m/20$.

has occurred to a greater or less degree in all individuals. Some of these, however, gradually undergo more or less recovery in water and finally attain the condition of Figures 59 and 60. These, too, raise the question of possible re-ectodermization of the apical region. After 18-19 hours in hypertonic $m/20$ the condition ranges from that of Figure 58 to forms like Figures 61 and 62 and to some with complete obliteration of the blastocoel. With this continued exposure to the hypertonic solution there is in general less, and often no, actual dissociation into free cells; probably because of the high salt content; but epithelial pattern may completely disappear. Returned to water at this time, individuals stick together in indefinite numbers on contact and give rise to large masses—at first irregular in form, but gradually becoming or approaching spherical, bounded by an epithelium and filled with cell debris or cells, some of which often develop pigment or epithelial vesicles. Single individuals become spherical and anaxiate like Figure 56, some with others without epithelial vesicles in the blastocoel; they differ from the large multiple masses only in size. Axiate pattern appears to be completely obliterated in these cases.

LITHIUM MODIFICATIONS WITH DIFFERENT EXPOSURE PERIODS
BEGINNING AT LATER STAGES

It has long been known that, with a given concentration, exposures beginning at the earliest developmental stages are most effective in producing exogastrulation. A few data concerning effects of late exposures are given here in order to direct attention to certain facts.

With continuous exposure to $m/60$ beginning at first cleavage hypertonic sea-water solutions give 60–70 per cent entexogastrulae or exogastrulae with more or less ento-



FIGS. 63–68.—Exposure from 5-hour blastula (Figs. 63–67) and from stage immediately preceding gastrulation (Fig. 68) to $m/45$ and $m/40$. Fig. 63, 14 hours in $m/45$, 24 hours in water; Figs. 64 and 65, 40 hours in $m/45$, 24 hours in water; Fig. 66, exogastrula, 14 hours in $m/40$, 25 hours in water; Fig. 67, outline from anal side of ectoderm of an exogastrula of the same lot as Fig. 66, indicating differential recovery; Fig. 68, condition after 11 hours in $m/40$ from stage immediately preceding gastrulation; entoderm more inhibited than ectoderm.

dermization of ectoderm. Similar exposure to approximately isotonic solution of the same concentration entodermizes slightly or not appreciably; more or less invagination of the original entoderm occurs in all; and only some 10–20 per cent are entexogastrulae. Incidentally, this difference suggests that hypertonicity increases entodermization. With exposure to the same isotonic concentration from the beginning of mesenchyme immigration no entodermization and no exogastrulae, or in one series, 1–2 per cent, result. In isotonic $m/50$ continuously from first cleavage, more or less entodermization up to the basal half of the prospective ectoderm and exogastrulation occur in 85–100 per cent; but half or more of these are entexogastrulae with further development of the invaginated entoderm inhibited. With continuous exposure to the same concentration from the 6-hour blastula there is no appreciable entodermization, 98–99 per cent invaginate and develop into somewhat differentially inhibited plutei. The remainder are exogas-

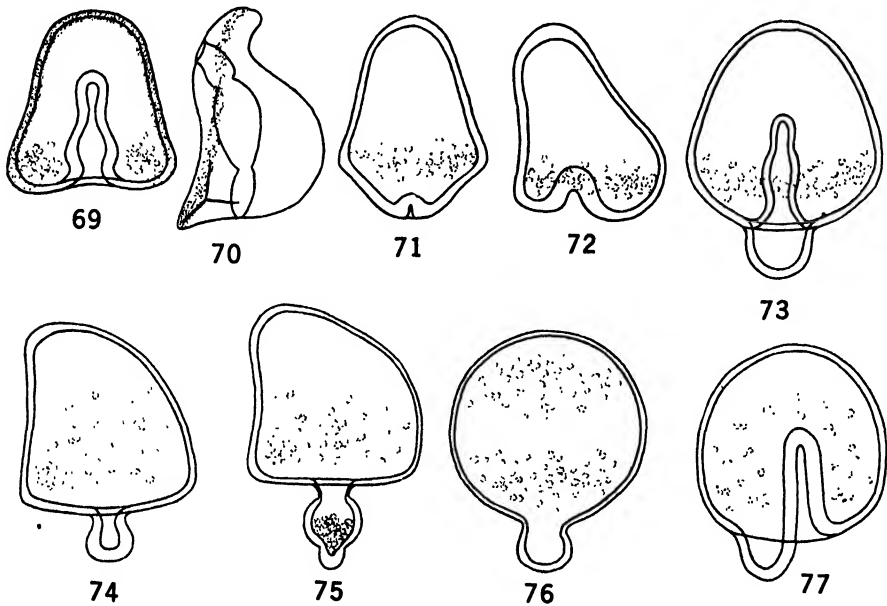
trulae but with small entoderm like those shown in following figures. Results are much the same with exposures of 14-40 hours to isotonic $m/45$ from the 5- to 6-hour blastula except that frequency of exogastrulae with small entoderm becomes higher. In these (Figs. 63-65) the entoderm remains small, shows little or no regional differentiation, while ectoderm develops ciliated band and sometimes stomodeal invagination (Fig. 64); and some individuals show secondary elongation of the apical region, indicating differential recovery (Fig. 63).

With 18 hours from first cleavage in isotonic $m/40$ and decrease in concentration to $m/160$ at that time, more or less entodermization results, and 100 per cent exogastrulae develop with large entoderm and with ectoderm ranging from differentially inhibited pluteus forms to completely radial in about 50 per cent. With 14 hours' exposure, beginning at the 5- to 6-hour blastula, entogastrulation and inhibited pluteus forms with more or less secondary modification by differential recovery are characteristic with a small percentage of exogastrulae of the type shown in Figure 66. The dorsally bent oral lobe is a very common feature of differential recovery following the less extreme inhibitions. The large evaginated entoderm, consisting wholly or almost wholly of the original prospective entoderm, also indicates differential recovery; at the end of the exposure period it was inhibited. Figure 67 shows the ectodermal outline viewed from the anal side in an exogastrula of the same type as Figure 66. Comparison of these results with those for $m/45$ above shows a greater degree of inhibition and less recovery for the same exposure period beginning at the same stage with the lower concentration. This is actually possible and has been observed not infrequently. During exposure the higher concentration inhibits development more than the lower, so that with the same exposure periods the return to water from the higher concentration occurs at an earlier stage of development than from the lower, and with the lesser degrees of inhibition the secondary modifications of differential recovery from the higher concentration may be greater than from the lower. Exposure to $m/40$ at beginning invagination does not produce exogastrulation in any case observed but does inhibit further development of entoderm to a greater extent than that of ectoderm. Secondary thickening and elongation of the apical region and development of the ciliated band take place while the entoderm remains without any regional differentiation (Fig. 68). Similar cases were described in an earlier paper (Child, 1936b).

As noted above, exposure to $m/30$ or $m/25$ for 5 hours or more from first cleavage is highly effective in producing exogastrulation with extensive entodermization, and with longer periods there is more or less dissociation of the original entoderm. The characteristic condition after 23 hours in $m/30$ from beginning gastrulation is shown in Figure 69. There is no entodermization and no exogastrulation; the invaginated entoderm is inhibited in development, particularly its apical region; but the ectoderm has developed a flat ventral side, and the ciliated band is distinguishable except ventrally. After return to water at this time, development is very slow; but 2 days later these forms have become small inhibited plutei with flat or somewhat convex ventral side, dorsally bent oral lobe, much inhibited, and short anal arms developing at wide angles up to 180° (Fig. 70). Slight differential recovery has occurred apicoventrally in the ectoderm and in the apical entoderm. Except for additional skeletal rods or spicules in some individuals, further development has not been observed.

Nine hours' exposure to $m/25$ from a stage immediately preceding gastrulation stops invagination in early stages (Figs. 71 and 72) or in the most advanced, with entoderm

halfway to the apical pole. With continued exposure, gradual dissociation and death result without further development. With decrease of concentration to $m/100$ after 9 hours' exposure 40–50 per cent become entexogastrulae or exogastrulae with small entoderm, usually with more or less ectodermal ventrodorsality, but some with completely spherical ectoderm (Figs. 73–76). Some entodermal dissociation is evident in Figure 75, and the cells in the basal region of the blastocoel in Figure 76 are probably dissociated from entoderm, but in the lower concentration the entoderm has recovered



FIGS. 69–77.—Exposure to $m/30$ and $m/25$ immediately preceding or at beginning of gastrulation. Fig. 69, 23 hours in $m/30$ from beginning gastrulation, with ciliated band incomplete basally; Fig. 70, 23 hours in $m/30$ from early gastrula; Figs. 71 and 72, inhibition of invagination with 9 hours' exposure to $m/25$ from stage immediately preceding gastrulation; Figs. 73–76, 9 hours in $m/25$ from stage immediately preceding gastrulation, then in $m/100$; Fig. 77, entexogastrulation in recovery after 24 hours in $m/25$ from beginning gastrulation.

to some extent and ceased to dissociate. The ventral side remains almost flat; no oral lobe, no arms (or only the earliest stages), and no stomodeum develop; and the entoderm remains blind and undifferentiated apically.

On return to water after 23 hours in $m/25$ from beginning gastrulation more or less recovery may occur. Some 20–30 per cent swimming at the surface may become small, differentially inhibited plutei. At the bottom 50–60 per cent remain differentially inhibited gastrulae, and 20 per cent become entexogastrulae or exogastrulae like Figures 73–77. In some of these with ventrodorsal difference in form the external entoderm is ventral to the invaginated, but whether this is always the case is uncertain in absence of visible ventrodorsality (Fig. 77).

VARIATIONS IN SUSCEPTIBILITY IN *Dendraster*

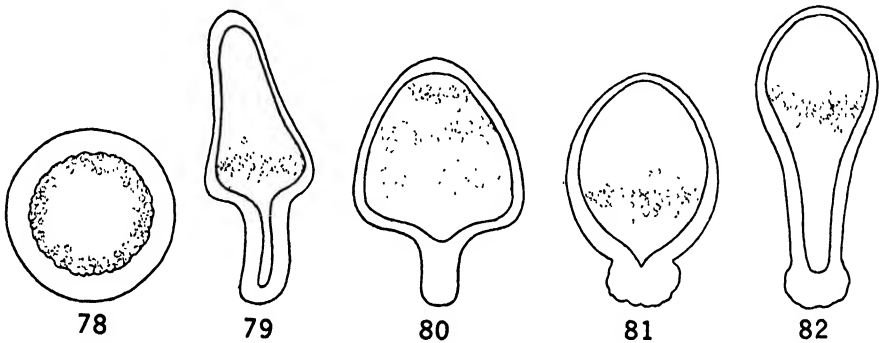
It is well known that even in a lot of eggs from a single female, fertilized by sperm from a single male, developmental modifications by lithium and by other agents always differ more or less in degree in different individuals. With certain procedures 100 per cent exogastrulae result, but these differ in degree of modification. With less extreme effects the resulting forms may range from plutei of normal or almost normal proportions through various degrees of differential inhibition, with perhaps more or less secondary modification, to a variable percentage of exogastrulae, or even inhibited gastrulae or blastulae. Also, with different lots of eggs or embryos the same lithium concentration and exposure period at the same developmental stage usually produce a certain range of modifications, and the same is true for other agents. By no means all these differences, however, result from inherent individual differences in susceptibility to lithium or other agents. Aggregations on the bottom of the container result in different environmental conditions from those to which more completely isolated individuals are subjected, and in the aggregation conditions as regards oxygen and CO₂ and perhaps other products of metabolism may differ from the center to the periphery. Animals from such aggregations often show more extreme modifications than those more completely isolated. Aggregations of blastulae and gastrulae at the surface of the solution probably also have some effect on degree of modification. It is of interest in this connection that in aggregations of animals stained with methylene blue or Janus green reduction of dye often occurs without experimental decrease of oxygen. Even a few individuals close together may reduce more rapidly in regions in contact with others or toward the center of the group. On the other hand, if aggregations occur early in the exposure period before the agent has attained its full effect, dilution of the agent about the aggregation may result; and, if lack of oxygen or other incidental factors do not become effective, the aggregated individuals may be less modified than others. Here, again, results with dyes are of interest; aggregations become less deeply stained than isolated individuals in dye solutions. In short, without continuous agitation a rather wide range of environmental conditions is likely to arise in a container with developing animals. That such differences in environment may be important factors in determining different degrees of modification in the same container is indicated by the effects of crowding considered below. In general, the smaller the number of animals in a given volume of water or solution, the less extreme are these differences likely to be; but even under these conditions, environment is not necessarily uniform. That inherent differences in susceptibility are absent is not maintained, though the uniformity in rate of development in lots under good environmental conditions suggests that they are not ordinarily very great; and it is sufficiently evident that relatively slight differences in environment acting at a certain developmental stage may bring about considerable differences in course of development.

ENTODERMIZATION BY OTHER AGENTS

A considerable range of crowding of a large number of individuals in a small volume of water during early stages is highly effective in producing differential inhibitions of development which do not differ in any essential respect from the inhibitions produced by lithium. Differential inhibition of ectoderm, increasing inhibition of entoderm with approach of gastrulation, dissociation of the original entoderm, entodermization of prospective ectoderm, entexogastrulae, and exogastrulae are characteristic effects of

crowding. Here only a few data are presented; extended comparative study of effects of different degrees of crowding has not yet been made.

With 16 hours' crowding from first cleavage the less affected are somewhat inhibited blastulae with the thick-walled basal region thicker and more extensive than in controls, suggesting entodermization. From these the forms range from early exogastrulae through blastulae with greatly thickened and extensive basal region from which cells are dissociating into the blastocoel, to spherical uniformly thick-walled blastulae with cells dissociating from the whole inner surface (Fig. 78), apparently completely anaxiate, and 10-20 per cent dead. A part of this lot taken from a layer at the bottom of the crowded container was returned to water after 16 hours' crowding. After 6½ hours in water 75 per cent of the living show more or less entodermization and develop as exogastrulae,



FIGS. 78-82.—Developmental modifications resulting from crowding and recovery. Fig. 78, apparently anaxiate form with dissociation from entire inner surface after 16 hours from first cleavage in crowded culture; Fig. 79, exogastrula with some entodermization after 16 hours' crowding from first cleavage and 6½ hours in water; Fig. 80, exogastrula, 16 hours' crowding from first cleavage, 6½ hours in water, with mesenchyme localized at three levels. Fig. 81, early exogastrula with entodermization, 10 hours' crowding from early blastula; Fig. 82, exogastrula with entodermization, 10 hours' crowding from early blastula, 5½ hours in water.

usually with some degree of differential recovery of the apical ectodermal region and of the entoderm (Fig. 79). They finally become exogastrulae with ectoderm approaching more or less normal pluteus form and with long external entoderm, usually with three regions more or less differentiated. Individuals not infrequently show two distinct circles of cells, supposedly mesenchyme, at different levels in the blastocoel; and in a few animals indications of a third circle have been observed (Fig. 80). Since this condition has been seen, not during the period of crowding but during that of recovery, it suggests the possibility that the cell circles may represent progress in re-ectodermization of prospective ectoderm entodermized during the period of crowding. In most individuals an excess of cells is given off from a more extensive basal region than that of mesenchyme immigration under natural conditions. Those reaching a level near the apical pole may be localized at one stage; those farther basally, at another stage later. However, further investigation of these forms is desirable. The blastulae swimming at the surface in the same crowded lot, continued under crowded conditions up to 22 hours from first cleavage, gave only about 10 per cent exogastrulae, the remainder developing as plutei more or less modified by differential inhibition and recovery and similar to lithium forms.

In another experiment after 10 hours' crowding from 6-hour blastula the level of mesenchyme localization indicated more or less entodermization in almost all individuals; and 50 per cent were already exogastrulae, usually with the original prospective entoderm a solid mass (Fig. 81). After $5\frac{1}{2}$ hours in well-aerated water, 90-95 per cent were exogastrulae with the original entoderm, or most of it, forming a solid cell mass at the tip of the entodermized ectoderm in most of them (Fig. 82). Later ventrodorsality and finally inhibited pluteus forms with more or less apicoventral recovery developed from the remaining ectoderm, and the external entoderm increased in length and attained some regional differentiation. As far as could be determined, *Dendraster* exogastrulae resulting from crowding do not differ essentially in any way from those produced by lithium. The differential inhibitions are the same, and the secondary modifications of recovery show the same differentials.

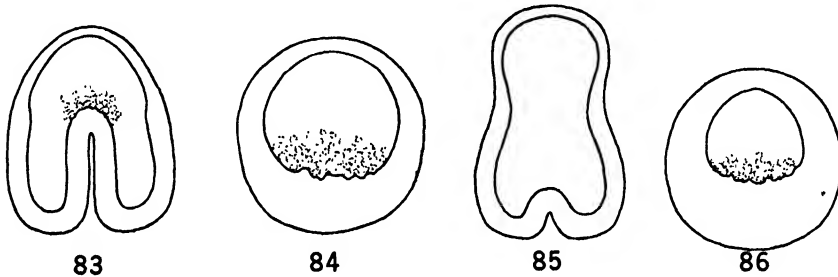
Exposure of *Patiria* embryos to low concentrations of Janus green, beginning at first cleavage or even with blastulae at the beginning of movement, retards greatly or completely inhibits invagination, brings about dissociation of cells from entoderm, and entodermizes prospective ectoderm. However, with concentrations sufficient to produce these effects, most of the dye in the cells remains there, even after repeated washings; and since it is highly toxic, there is little or no recovery after return to water, and development does not proceed far beyond the stage of return.

With long-time exposures to very low concentrations of this dye the concentration of dye in solution decreases as it is taken up by the animals. In lots consisting of approximately standard numbers of animals in standard solution volumes of concentrations of $1/2,000,000$ or less, all—or practically all—visible dye is removed from the solution by the animals within 30-40 hours; consequently, maximum accumulation in the cells must be approached or attained at that time or earlier.

Patiria embryos in Janus green, approximately $1/2,000,000$ in initial concentration from first cleavage show, after 42 hours, great retardation of development in the living, and some 50 per cent are dead. In the least inhibited, ectoderm has not attained normal dimensions, entoderm is less inhibited than ectoderm and may invaginate and develop to the stage of apical enlargement, but dissociation of cells from its apical region is much more extensive than in normal formation of mesenchyme. In low oxygen, dye reduction occurs more rapidly in the invaginated entoderm than in the ectoderm; but the gradient in rate of reduction decreases acropetally instead of basipetally, as in controls (Child, 1936a), suggesting a differential inhibition, greatest in the apical entoderm and decreasing basipetally, as might be expected. The more affected individuals are smaller, invagination has stopped in early stages or has not begun, and there is more or less entodermization of prospective ectoderm and dissociation of cells from the original entoderm (Figs. 83 and 84). With this experimental procedure development stopped at these stages.

In another series in Janus green $1/2,000,000$ for 30 hours from the blastula stage beginning movement, 40-50 per cent were essentially similar to Figure 85. In many of these cytolysis of the apical region was beginning. Others show somewhat more invagination and less entodermization. Death occurs with little or no further development. After repeated washings and 36 hours in water following exposure of early cleavage stages for 10 minutes to Janus green $1/50,000$, invagination of entoderm does not take place; but the apicobasal differential in thickness of the blastula wall is greater than in controls, an extensive basal region becoming very thick, often with dissociation of

cells into the blastocoel. The more extreme modifications are like Figures 84 and 86. There is no further development. Dissociation of cells from the basal region is very generally characteristic of inhibiting conditions in asteroids as well as in echinoids. Also, the extension of the thick-walled region acropetally suggests entodermization. If recovery and further development were possible, many of these forms would undoubtedly become exogastrulae. With the higher concentrations of LiCl earlier stages of *Patiria* and *Dendroaster* exogastrulae are similar to these forms, except that the apicobasal differential in effect is often greater with lithium (see Figs. 53, 54, 57, 58, 61, and 62).



FIGS. 83-86.—*Patiria*: developmental modification by Janus green. Figs. 83 and 84, inhibited invagination of entoderm, dissociation of cells, and entodermization of prospective ectoderm after 42 hours from first cleavage in Janus green, initial concentration approximately $1/2,000,000$; Fig. 85, entexogastrula with inhibited invagination of original entoderm and entodermization of ectoderm after 30 hours in Janus green, initial concentration approximately $1/2,000,000$, from blastula stage beginning movement; Fig. 86, 10 minutes in Janus green, approximately $1/50,000$ at early blastula, 36 hours in water.

DISCUSSION

Lithium chloride is highly effective in bringing about differential modification of echinoderm development, apparently because very considerable regional difference in degree of its inhibiting action may occur without death of any part. In other words, the regional difference along an axis in susceptibility to LiCl appears to be greater than to various other agents.

Inhibitory ectodermal modifications.—With exposure to effective ranges of lithium concentration, beginning shortly after fertilization, differential inhibition decreases from the apical or animal pole basipetally. The apical region remains relatively smaller, and its wall often thicker, than under natural conditions, while the basal region attains more nearly normal size in blastula stages. Comparison of Figure 1, normal, with Figures 15, 22, 28, and 29 shows this differential effect. With adequate concentrations and exposure periods ectodermal differential inhibition becomes apicoventral as development progresses. All degrees of inhibition of the oral lobe developing from the apical region and of the ventral side appear, resulting in modifications with oral lobe reduced or absent, and with a flat, or even convex, ventral side (Figs. 13, 14, 32, 33, and 46). As regards inhibition of the ventral side, different modifications result from different degrees of inhibition. Decrease of arm angle (Fig. 40), parallel or more or less fused arms (Fig. 21), or one-armed forms (Figs. 25, 26, 34, 37-39) may result. When exposure is continued or the inhibiting effect persists to later postgastrular stages, origin of local organ gradients of oral lobe and arms is more or less completely inhibited. With the more extreme

differential inhibitions ventrodorsality may be completely obliterated, the ectoderm remaining completely radial, even after return to water.⁴ With obliteration of ventrodorsality the mesenchyme does not become bilaterally localized. These differential inhibitions of ventrodorsality are entirely similar in principle to the modifications by differential inhibition with various agents of the planarian head,⁵ the heads of fish embryos,⁶ and amphibia (Bellamy, 1919). In those forms with increasing inhibition bilaterally localized organs of the cephalic region develop progressively nearer the median plane, become single in the median plane, or are entirely absent. The single apical coelom (Fig. 46) represents a similar mediolateral differential inhibition. With extreme differential inhibition axiate pattern may be completely obliterated from the ectoderm as far as any differentiation is concerned (Figs. 49, 51, 52, and 60). In these cases, however, the remaining ectoderm is only the apical part of the original prospective ectoderm.

Inhibitory modifications of prospective entoderm.—Although prospective entoderm is less inhibited than apical regions in earlier pregastrular stages, it becomes more susceptible as gastrulation approaches. If it invaginates, its further development is more or less inhibited, its apical region most of all. Coelom development may not occur, or a single apical coelom may develop without any evidence of bilaterality (Fig. 46). This is evidently another case of mediolateral differential inhibition similar to those of the anal arms, the planarian, the fish, and the amphibian head. Dissociation of cells from its more apical levels and extending basipetally may take place (Figs. 14, 16, 17, 32), or its apical portion may become a solid cell mass (Fig. 45), while ectodermal development proceeds further. With more extreme lithium action dissociation begins without invagination or with evagination. Parts of the prospective entoderm remaining undissociated appear to lose in part or wholly their epithelial arrangement and become a solid mass; or most or all of the entoderm may dissociate internally, externally, or both.⁷ The solid cell masses at the entodermal tips of many exogastrulae are evidently parts of the original prospective entoderm which have not yet dissociated (Figs. 18, 41, 44, 48, and 49). The thick-walled tips of the entoderm of many exogastrulae may also be parts of the original entoderm with epithelial order retained or regained (Figs. 37, 39, and 47). In very high concentrations the loss of epithelial order progresses acropetally until the whole individual becomes a solid mass (Figs. 58, 61, and 62).

With a certain range of concentrations and with exposure periods beginning in later pregastrular stages or immediately before gastrulation, the original entoderm may invaginate or evaginate partially or wholly but is more inhibited in development than ectoderm (Figs. 63–65, 68, 69, 71–77). The evidence indicates that with approach of gastrulation the prospective entoderm undergoes a change in physiological condition rendering it more susceptible to lithium action than in earlier stages and more susceptible than ectoderm. This evidence agrees with that from differential dye reduction in low oxygen in indicating that prospective entoderm, as well as mesenchyme, undergoes a considerable activation preceding gastrulation. If this is the case, increase in susceptibility to lithium is to be expected.

⁴ See Figs. 18, 24, 27, 30, 31, 35, 36, 41, 43, 44, 47, 48, 49, 51, 52, 59, and 60.

⁵ Child, 1921, and earlier papers; Child and Watanabe, 1935.

⁶ Stockard, 1907a, 1907b, 1909, 1910, 1914, 1921.

⁷ See Figs. 15, 22–24, 28–31, 33–37, 39, 41, 42–44, 47–60.

Entodermization of prospective ectoderm.—That exposure to lithium in early stages may transform more or less, or in extreme cases all, of the prospective ectoderm into entoderm which constitutes a larger or smaller part of the external entoderm of exogastrulae appears evident. The question how this entodermization is brought about and whether it represents a specific lithium effect is of considerable interest. As already pointed out, Runnström and his co-workers regard lithium as enhancing and extending acropetally the effectiveness of a vegetal gradient, presumably by favoring or determining formation of the specific vegetal substance or substances postulated as constituting this gradient. With, or resulting from, this action there is a corresponding decrease in extent or at least in effectiveness of the opposed animal gradient. Actually, however, lithium appears to be a general inhibitor of development. It inhibits development of prospective entoderm, though in early stages less than that of prospective ectoderm, while at the same time it may entodermize prospective ectoderm. Moreover, as gastrulation approaches, prospective entoderm becomes more susceptible to, and so more inhibited by, lithium than ectoderm; but little or no entodermization occurs with exposure to lithium beginning in these more advanced stages. In short, lithium inhibits the original prospective entoderm, and entodermizes prospective ectoderm. It also inhibits more or less completely further development of entodermized ectoderm. In Figures 13, 14, and 16–18 there is considerable entodermization, but these figures represent the most advanced stages attained with continuous exposure to concentration of $m/70$ to $m/50$. Only after return from these concentrations to water at a stage near that of Figure 15 does entodermal development proceed farther. In higher concentrations inhibition of entodermized ectoderm, as well as of the original entoderm, is even more evident (Figs. 28, 29, 53, 54, 57, 58, 61, and 62). Development ceases early unless return to water or to a much lower lithium concentration occurs before the inhibiting action becomes irreversible.

The later in pregastrular development exposure to lithium begins, the less the degree of entodermization. With exposure beginning just preceding, or at the beginning of, gastrulation, there is little or no appreciable entodermization (Figs. 63–77), presumably because differentiation of ectoderm has attained a stage which makes it impossible under the conditions of the experiments; but further development is still more or less inhibited, and entoderm is more inhibited than ectoderm.

So far, then, it appears that lithium is generally inhibitory in its action on *Dendroaster* development. If this is the case, the question at once arises whether its action in entodermizing prospective ectoderm may not also be inhibitory. Lithium is not the only agent that entodermizes prospective ectoderm. Crowding in early stages also entodermizes and produces exogastrulation and loss of epithelial order in the original entoderm (Figs. 79–82). It also decreases and obliterates ventrodorsality and even polarity in the ectoderm and, with more extreme action, brings about dissociation of prospective entoderm exactly as does lithium. It may even bring about dissociation from all levels of blastulae (Fig. 78). This also results with high lithium concentrations. At present there is no evidence to indicate that crowding has any other than an inhibitory action, but its differential effects are similar to those produced by lithium. The incidental data on *Patiria* show that Janus green is a highly effective inhibitor of development. In early stages its inhibiting action decreases basipetally; but it inhibits invagination more or less completely, dissociates entoderm, entodermizes prospective ectoderm, and also gives rise to exogastrulae. Except that it is much more toxic than lithium and, in concentra-

tions within the cells sufficient to produce these modifications, stops development and kills in relatively early stages, its differential action on development is essentially similar to that of lithium. Here, again, there is no evidence to indicate anything but inhibitory action.

In view of these facts, it seems that we must assume either that crowding and Janus green have a specific effect on the vegetal gradient like that of lithium or that lithium, like crowding and Janus green, inhibits development generally but differentially and without regional specific action. In the light of all the evidence the second alternative appears the more probable.

If prospective entoderm originates from the lower levels of a primary apicobasal gradient, as dye reduction and differential lethal susceptibility indicate,⁸ entodermization may result from depression or inhibition of prospective ectoderm below a certain "physiological level"; that is, the specific difference between ectoderm and entoderm may be a secondary result of a nonspecific, primarily quantitative difference. According to this suggestion, entodermization occurs first in the most basal levels of prospective ectoderm and progresses acropetally with increasing inhibition because lower levels of ectoderm require only relatively slight inhibition, higher levels, more extreme inhibition, to bring them down to the entodermal level.

The question has already been raised whether the forms with external entoderm originating from entodermized ectoderm are, strictly speaking, exogastrulae. Under natural conditions the entodermized ectoderm would have developed as ectoderm and would never have invaginated. Entodermization apparently has no relation to gastrulation, for entodermized ectoderm shows no tendency to invaginate, even after return to water, though the original prospective entoderm very often invaginates after conditioning or recovery (Figs. 13, 14, 17, and 25). Actually, these forms are differential modifications of prospective ectoderm, more or less entodermized; the original prospective entoderm may or may not take part in their formation. We might assume that, when cells of prospective ectoderm become entoderm, they acquire a polarity which, under natural conditions, would result in invagination and that lithium and other entodermizing agents reverse or obliterate this polarity. If that were the case, we should expect that entodermized ectoderm would invaginate after return to water at a certain time; but such invagination has not been observed. It may even be questioned whether the entoderm of exogastrulae derived from entodermized ectoderm is inside out. It remains in essentially the same relation to other parts as when it was prospective ectoderm, and there is no indication that the polarity of its cells has been reversed. The only reason for believing that it is inside out is the fact that it is external instead of internal. However, in view of established usage and for convenience, forms with entodermized ectoderm may be called "exogastrulae" in a purely descriptive sense, because their entoderms are external; but it should not be forgotten that, as regards origin, they differ widely from true exogastrulae, in which the original entoderm evaginates instead of invaginates.

Mesenchyme.—Except in very high concentrations (Figs. 58, 61, and 62) mesenchyme immigrates; but only in slightly inhibiting concentrations does it become bilaterally localized during exposure, though this localization may follow return to water or may appear secondarily in conditioning. In individuals with entodermization mesenchyme is mostly localized in a circle at, or slightly apical to, the boundary between remaining

⁸ Child, 1936a, 1936b; see also Child, 1915, 1916a, 1916b.

ectoderm and entodermized ectoderm (Figs. 15, 22, 28-31, 42, 50, and 55). Cells dissociated into the blastocoel from entoderm resemble mesenchyme, but whether they play any part in further development is uncertain. Perhaps some of them may develop as mesenchyme; but most, if not all of them, usually appear not to develop further except when they form entodermal vesicles in recovery (Figs. 51, 55, and 56). With obliteration of ventrodorsality mesenchyme remains in a circle; and, if skeleton develops, it is merely an irregular circle of spicules (Figs. 35 and 41). With further reduction of ectoderm mesenchyme is merely aggregated without order in the ectodermal blastocoel and sometimes with recovery develops a single rod, often longer than the ectoderm (Figs. 48, 49, 51, 52, 59, and 60). With complete inhibition of skeletal development abundant pigment may still develop. The small ectodermal regions of the more extreme exogastrulae (Figs. 48, 51, 52, and 60) usually become heavily pigmented (not shown in figures). Normal skeletal pattern develops only in very low concentrations or in recovery after short exposure. In general, with increasing inhibition skeletal development becomes increasingly aberrant and incomplete or absent. Evidently orienting factors in the ectoderm become less effective or are obliterated, and the mesenchyme cells become less able to form skeleton, though they may regain the ability to some extent on recovery.

Secondary modifications.—With continuous exposure from early stages to a certain range of low concentrations development is primarily retarded with slight differential inhibition; but a differential tolerance, acclimation, or conditioning may gradually become evident in secondary differential modifications opposite in direction to the primary differential inhibitions. After return to water following exposure, even to strongly inhibiting concentrations, modifications similar in character but often greater in degree may result. After slight inhibitions the secondary modifications may be wide-angled plutei with large oral lobes and small body, either in differential conditioning (Fig. 6) or in differential recovery. These are, in general, similar to secondary modifications in *Arbacia* obtained with other agents (Child, 1916b). With inhibition sufficient to produce exogastrulae differential tolerance, conditioning (Figs. 13 and 14) or recovery (Figs. 32, 33, 46, and 67) may result in more or less ectodermal modification.

Secondary modifications also occur in the entoderms of exogastrulae. For example, in $m/35$ from first cleavage development does not proceed far beyond stages shown in Figures 22 and 23; but after return to water, forms like Figures 24-27 develop. Figures 28-31 show development with continuous exposure to $m/30$; Figures 32-36, forms developing in water after various exposures to $m/30$. Figures 45-49 show differential recovery from $m/25$, a still more inhibiting concentration with continuous exposure. The very large entoderms of the more extreme exogastrulae (e.g., Figs. 33-37, 39, 41, 47-49, 59, and 60) are not direct effects of lithium but are results of differential recovery from lithium inhibition. Some relative increase in size of entoderm may appear in differential tolerance or conditioning (Fig. 18), but development of exogastrular entoderm is greatest in recovery, and most or all of this entoderm may be entodermized ectoderm. Apparently entodermization may be followed after return to water by more or less activation of the entodermized region: this is also indicated by dye reduction (Child, 1936b).

Entoderm which has become a solid cell mass may apparently regain epithelial order to some extent with recovery. In water after inhibition by high concentrations, epithelial vesicles often appear in the blastocoel (Figs. 51, 55, and 56), though not present during exposure. Also, the solid masses at the entodermal tips of exogastrulae not infrequently appear gradually to become epithelial with continued life in water. Develop-

ment in recovery of forms like Figures 59 and 60 from individuals like Figures 57 and 58 shows how great the secondary modification of recovery may be. With continued exposure to lithium death results without development beyond the stage of Figures 57 and 58. However, in these particular cases development of single individuals has not been followed through.

There are many indications that prospective ectoderm after entodermization or approach to entodermal condition may finally develop as ectoderm in secondary modification. In many exogastrulae with more or less skeletal development after return to water, the skeleton, instead of being localized about the anal side of the ectoderm, forms nearer the apical pole (Figs. 19, 35, 37, and 41). Also, both in differential tolerance or in conditioning in low concentrations, and in differential recovery, arms often develop at levels farther apical in the ectoderm than their normal position and the ectodermal region basal to the arm-level is enlarged and convex (Figs. 13, 14, and 33). In many exogastrulae without skeletal development the circle of mesenchyme is localized much farther apical than its usual position, even near the apical pole (Figs. 36, 44, 47, and 55). Moreover, two distinct circles of mesenchyme appear in some individuals; and in a case from a crowded lot, indication of a third circle was observed (Fig. 80). These cases suggest a primary entodermization sufficient to determine localization of mesenchyme and, with secondary modification, a re-ectodermization of the more apical levels. However, if this is the case, the mesenchyme does not migrate basipetally but remains at the level determined by the primary entodermization. In those individuals with two or three circles at different levels part of the mesenchyme may have been localized at different stages of entodermization or re-ectodermization, perhaps with increase in cell number during recovery. If inhibition or depression, rather than formation of a specific substance or substances, is the primary factor in entodermization, it seems probable that, if the inhibiting factor is removed early enough, before definitive differentiation, the cells concerned may regain or approach their original condition. Since return or approach to the original condition certainly does take place in many cells with tolerance, conditioning, or recovery after primary inhibition by lithium and other agents, its occurrence here presents no difficulties. Re-ectodermization has been reported by von Ubisch (1925, 1929) in a sea urchin following lithium treatment with isolation of parts.

Mesenchyme may fail completely to develop skeleton in LiCl but may regain its ability to do so after return to water, but form and distribution of skeleton differs widely with degree of axiate pattern present in the ectoderm. With differential recovery from less extreme inhibitions excess of skeleton may develop with formation of three, four, and even five arms or an indefinite accumulation of spicules and rods in the body. The relative roles of ectoderm and skeleton in development of supernumerary arms have not been determined; but in the cases observed, the ciliated band and the arms develop in the usual relation.

In general, regions primarily most inhibited by lithium show secondarily the greatest development in differential conditioning and recovery unless they have been so greatly inhibited that secondary modification becomes impossible before development has attained its most advanced stage. The forms resulting depend on exposure period as well as on concentration. The local activations associated with development of oral lobe and anal arms take place normally at certain stages of development. With exposures beginning in early stages the apical region may be so inhibited that an oral lobe is com-

pletely absent, even with recovery; but more or less arm development may still be possible after return to water or with conditioning.

Reconstitution in exogastrulae.—In those exogastrulae with partial entodermization of ectoderm, development of the remaining ectoderm involves extensive reconstitution, except when axiate ectodermal pattern is completely obliterated, as in Figures 27, 43, 49, 51, 52, and 60. Scale of organization decreases with decrease in size of ectoderm, and everywhere except in the extreme apical region the various parts differentiate in cells which normally would differentiate otherwise. A modified pluteus body may differentiate from the apical half, or even less than half, of the original prospective ectoderm. In these cases the anal arms develop from regions which normally would give rise to parts intermediate between arms and oral lobe. Whether or to what extent localization and outgrowth of arms are determined by skeletal development or by ectoderm in these forms is not known. In normal development the ectoderm is concerned in localizing the mesenchyme, but in these partial ectoderms the arms develop from ectoderm of quite different prospective significance. Since ventrodorsality is still present and arms develop in definite relation to it in these cases, it appears probable that even these partial ectoderms play some part in localizing mesenchyme and skeleton. However, in some exogastrulae with a single longitudinal skeletal rod, as in Figures 52 and 59, a short armlike outgrowth with the skeletal rod extending to its tip may develop from the apical pole; but it may be questioned whether this is, strictly speaking, an arm or merely an outpushing of the ectoderm by elongation of the skeletal rod.

Although isolated apical halves of early stages do not gastrulate and develop complete individuals under natural conditions (Hörstadius, 1935), it is evident that less than an apical half of the ectoderm in exogastrulae may develop parts normally arising from regions farther basal and may approach more or less pluteus form. It was shown by von Ubisch (1925) that apical halves, after treatment with lithium, may even develop entoderm and gastrulate. If these ectodermal reconstitutions in exogastrulae and isolated apical halves were due to increase of a vegetal gradient of specific substance or of some other kind, we should expect to find more extensive reconstitution of basal (vegetal) regions by the remaining ectoderm of exogastrulae with increase of lithium effect, at least up to a certain limit; but actually such development decreases. Development of arms is inhibited, ventrodorsality and even polarity do not appear or are obliterated with increasing lithium action; but with recovery, even from exposure periods and concentrations which stop development in its earlier stages, more or less ectodermal reconstitution may take place.

In terms of the gradient concept advanced in this paper, reconstitution of more basal body-levels by partial prospective ectoderms of exogastrulae and isolated apical halves results from differential inhibition with shortening and lowering of physiological levels of the primary apicobasal gradient, with consequent decrease in scale of organization, so that the partial ectoderm approaches, or in the isolated half may attain, wholeness in development. This effect is not specific for lithium; crowding in *Dendraster* and other agents with other species give essentially the same results. The alteration of the primary gradient is a direct effect of lithium or other agents; but the reconstitution, with further development than is possible in lithium, and origin of an archenteric gradient in isolated, lithium-treated halves and of arm gradients in exogastrular partial ectoderms, usually, if not always, involves secondary modifications resulting from differential tolerance, conditioning, or recovery, as clearly shown above.

Hydroid reconstitution shows essentially similar conditions. A piece of *Tubularia* or *Corymorpha* stem so short that under natural conditions it gives rise only to a hydranth or to the apical region of a hydranth may, after exposure to inhibiting agents, develop a smaller complete hydranth and stem, and in *Corymorpha* a basal holdfast region also. The gradient determining reconstitution is, under natural conditions, on such a scale that only its more apical levels are present in the short piece, and only a hydranth or the more apical levels of a hydranth develop. Inhibiting agents lower its levels differentially and shorten it; consequently, the scale of organization is decreased, and hydranth and stem or a complete individual on a smaller scale develop from the piece (Child, 1931, and unpublished data). Conversely, increase in scale of organization may result from elevation of temperature. Since these alterations in scale of organization in hydroids are produced by various agents, there is no ground for believing that they result from specific effect of a particular agent on a particular substance or substance gradient, and it does not appear at present that such a hypothesis is necessary to account for decrease in scale of organization in echinoderm development.

The small thin-walled "neck" often present between what would have been the anal region in normal development and the external entoderm (e.g., Figs. 14, 19, 24, 25, 27, 39, 41, 43, and 44, etc.) also represents a reconstitution and probably always a secondary modification. It is apparently ectodermal in origin, and the entoderm often separates from it after return to water, leaving it attached to the remaining ectoderm. If entodermized ectoderm is not entoderm inside out, its attached end may represent its apical end; the higher rate of dye reduction at this end in most exogastrulae of this type (Child, 1936b), as well as its origin from ectoderm, in which levels nearer the apical pole represent higher gradient levels, adds support to this suggestion. In this connection it is perhaps a point of some significance that the usually flattened basal region of the ectoderm in partially entodermized forms is usually the region of highest rate of dye reduction in the ectoderm, except when secondary apical modification and outgrowth occur (Child, 1936b); that is, in these exogastrulae the relations of the basal ectoderm to other parts are apparently more or less like those of regions about the apical pole in normal development. Under these conditions development of a stomodeum from the prospective anal region does not appear improbable. Reconstitucional development of a ciliated band in circular form around the basal region of radial exogastrular ectoderms (Figs. 24, 35, 41, and 44) is probably also associated with this change in the ectodermal gradients. With recovery after obliteration of ventrodorsality the physiological condition of the basal ectoderm may become enough like that of the normal ventral region to permit development of the ciliated band about its margin.

This neck may develop, however, in forms with little or no entodermization. In these the attached end of the entoderm is presumably the anal end; and the neck might be regarded as a proctodeum, developing under the experimental conditions, although absent or not developing appreciably under natural conditions. But it does not seem necessary to assume that it should always be either proctodeum or stomodeum: the important point is that it is a development of ectoderm, a reconstitution, resembling a proctodeum or stomodeum.

In exogastrulae with entoderm consisting in part or wholly of entodermized ectoderm, entodermal reconstitution is also involved. Entodermization is itself a reconstitution; but there is often further reconstitution, particularly with secondary modification. If the original prospective entoderm is not completely dissociated, it may form only the tip of

the exogastrular entoderm, instead of the whole; but development of the whole entoderm is orderly and definite, as far as it goes. With secondary modification, particularly with recovery, two or three entodermal regions, separated by constrictions, may develop (Figs. 19, 24, 33-35, 37, 39, 41, 47-49, 52, 59, and 60). This development takes place in part or wholly in tissue originally prospective ectoderm and evidently results from extensive and orderly reconstitution. The original entoderm, as far as it takes part, forms less than normally, and development of entodermized ectoderm differs completely from its normal fate. How such an orderly and harmonious development is determined in cells of very different prospective significance is an interesting question. If it were the result of increase in concentration and effective extent of a vegetal substance gradient, we should expect that the original prospective entoderm would develop more entoderm instead of less than normally. To account for this orderly and definite development, a definite pattern of relations between parts somewhat similar to that determining entodermal development in the normal animal seems necessary. In order that the original prospective entoderm, or some part of it, shall develop as merely a portion of exogastrular entoderm, its development must be controlled by factors in other parts. Development of regional pattern in exogastrular entoderm is more or less completely inhibited by lithium, except with differential tolerance or conditioning in low concentrations; only with recovery does it attain its fullest expression. With entodermization of the entire ectoderm, development of this pattern has never been seen in any individual of any species with which the writer has worked. Apparently the differences in physiological condition and the relations between parts which determine orderly axiate pattern may be completely obliterated in the original entoderm, in entodermized ectoderm, and in ectoderm by lithium and other inhibiting agents (Child, 1916b). If these relations originate in a physiological gradient or gradients, lithium and various other agents apparently obliterate the gradients by differential inhibition. If not completely obliterated, they may attain some degree of developmental expression with secondary modification. In exogastrulae the gradients and relations of parts associated with them are so altered that extensive reconstitution results.

THE PHYSIOLOGICAL-GRADIENT CONCEPT

The theory that axiate pattern in its simplest terms consists in a physiological gradient or gradient system in which differences in rate, rather than in kind, of metabolism are the primary active and effective factors in initiating and carrying on actual development does not assume that such gradient patterns are independent of a material substratum. It merely holds, first, that a gradient pattern may be initiated without pre-existing specific regional organization in the materials of the substrate in which it appears. The fact that physiological axes determining the reconstitution of individuals with a completely new organization can be established at practically any body-level in hydroids, planarians, and various other forms by a local activation or a purely quantitative external differential without definite relation to a pre-existing organization provides much evidence in support of this conclusion and none against it.

Second, the theory holds that differences in concentration of a multitude of substances must at once begin to appear at different levels of such a gradient in association with the different rates of reaction. Differences in rate of transformation of nutritive material and use of oxygen in relation to intake or presence in the protoplasm, in production of CO_2 and other metabolites, and in synthesis and breakdown of protoplasmic

components provide almost infinite possibilities for the origin of specific material differences at different levels of such a gradient. With these reactions and their products are associated differences in water content, in ionization of various electrolytes, and in factors associated with the colloid substrate. Even if a physiological gradient is purely quantitative at the moment of its establishment, it seems impossible that it should remain so for any considerable length of time. The theory maintains that such a gradient provides an adequate basis for definite future differentiations at its different levels, both through the specific organization resulting directly from it and as a factor making possible realization of different gene potentialities in different regions or cells. If "determination" of a part in the course of development represents attainment of a certain degree of chemical or structural specificity in that part, the question at once arises: How did that part differ from others preceding its determination for a particular developmental fate? It must have differed in some way; otherwise it could not become determined as a certain part or organ. Can the primary differences between it and other parts have been anything but quantitative? Experimental investigation of amphibian development, for example, shows progressively less evidence of specificity in different regions as we pass from later to earlier stages. This does not necessarily mean that the amphibian egg at the beginning of embryonic development possesses nothing but a quantitative gradient pattern; but it may mean that, if we could extend experimentation back into the period of ovarian development of the oöcyte, we should come finally to a quantitative pattern. Determination of a part in development appears to be a gradual process and in general becomes progressively more stable: this certainly indicates a progressive increase in specificity.

Objection to the concept of a primarily quantitative axiate pattern with rate of basal metabolism as the active, effective factor has been raised on the ground that, if this were the case, differential modification should result from change in temperature. The answer to this objection is that with a sufficient change in temperature such modification does occur in various organisms. Low temperature inhibits differentially reconstitution of the planarian head and may produce all degrees of the graded series of forms—teratophthalmic, teratomorphic, anophthalmic, and acephalic—in pieces which at higher temperatures develop normal heads. In these cases the differential inhibition is most conspicuous mediolaterally, inhibition decreasing laterally from the median region; but it also decreases from anterior to posterior levels. High temperature increases not only rate of reconstitution and size of head but also frequency of normal heads on pieces which develop differentially inhibited heads at lower temperatures. In some individuals it also determines heads with second right and left eyespots, each lateral to the normal one, with corresponding greater development laterally of the cephalic ganglia. Moreover, it may bring about metamorphosis of teratomorphic heads with a single median eyespot and cephalic lobes approximated to, or in, the median plane, into heads with two eyespots in normal position right and left of the median plane in addition to the median eyespot, reduction of the cephalic lobes near or in the median plane anteriorly, and development of new lobes right and left in normal position; also, increased development of the cephalic ganglia. Animals conditioned to low temperature before section reconstitute normal heads in higher frequency when temperature is raised following section than those not so conditioned; and with conditioning to high temperature before section, the frequency of differentially inhibited heads is higher at low temperature than in pieces from animals not so conditioned. With recovery from the differential inhibition of low

temperature, further development of differentially inhibited head-forms may occur with more or less approach to normal.⁹ Differential modifications of echinoderm development have also been produced by change of temperature (unpublished).

However, the possibilities of developmental modification by temperature have not been extensively explored. Amphibian embryos have been subjected to temperature gradients, and effects of different temperatures on development of the fish and chick have been recorded; but really analytic investigation of temperature in this relation to embryonic development has not yet been undertaken. It is perhaps not necessary to point out that, after specific regional "determinations" have originated in an egg or embryo, differential modification by temperature may still be possible to some extent because quantitative gradient factors do not necessarily disappear with occurrence of determination; but we might expect the modifications to be less extreme than in stages preceding determination and different in character as new gradients appear. In *Dendraster* the most extreme lithium modifications are obtained with exposure at the earliest stages, but differential modifications are still possible with exposure beginning in gastrula stages when ectoderm and entoderm are apparently rather stably determined. To produce differential modification a gradient must apparently be differentially altered; the differentials of different levels must be either increased or decreased. Investigation of temperature coefficients of chemical reactions indicates that they do not remain constant at all temperatures, even within the physiological range; and temperatures above and below this range become differentially injurious or lethal. Certain difficulties are associated with use of low temperature for differential developmental modification. In planarian pieces, for example, conditioning to low temperature may occur, even though development is practically at a standstill; consequently, there may finally be less differential inhibition than expected. Also, low temperatures may approach or fall below the threshold of development; and temperatures that freeze the organism are, of course, useless for experiment of this sort. High temperatures beyond a certain limit are inhibitory or lethal, except as conditioning may take place. The chief difference between temperature and other physical and chemical agents as regards differential action on development seems to be the range within which differential modification does not appear or is slight. Two factors, and perhaps others, may be concerned in determining this range. First, within it the whole gradient system may be elevated or lowered without much alteration of the differentials of different levels. There is no reason for believing that equal change in rate of reaction in the whole system will modify development differentially, though it will accelerate or retard, and may increase, scale of organization. Second, changes in temperature are features of natural environment, and most organisms have attained a relatively wide range of tolerance and become rapidly conditioned to these changes or recover rapidly from them; consequently they produce little or no modification.

As a third point, the gradient theory holds that, even though specific regional differences are already present to some extent along a gradient, differences in rate of the basal metabolic reactions may still be important factors in determining form and proportion, and perhaps even course of differentiation. These differences in rate may be altered nonspecifically by external agents, whatever the particular way in which a certain agent brings about the alteration; and differential modification of form and proportion may result even though determination of parts is already advanced.

Fourth, the theory does not maintain that axiate pattern of eggs at the beginning of

⁹ Child, 1912, and unpublished data; Behre, 1918.

embryonic development is always the purely quantitative primary pattern. Obviously, it is not. The egg is a more or less highly specialized cell, and in many eggs a considerable regional differentiation is already present at the first cleavage. Even in some of those, however, quantitative gradient differences are still present and are essential factors in development, and extensive differential modification of development is still possible. The interpretation of exogastrulation in the present paper does not, by any means, exclude the possibility that the two opposed specific concentration gradients postulated by Runnström are present in the echinoderm egg. It is merely based on evidence indicating that, whether they are or are not present, lithium does not act specifically or exclusively on one of them but is rather a differential inhibitor of development in general, the degree of inhibition depending, in some unknown way, on difference in rate of vital activity rather than on concentrations of specific substances. Concentrations in themselves without metabolism can accomplish nothing in development; and two opposed concentration gradients, such as Runnström postulates, may be associated with, or result from, a single gradient in rate of metabolism; that is, a single gradient in rate of metabolism may coexist with decrease of one and increase of another substance gradient in a certain direction. The evidence from differential modification of development and differential dye reduction indicates a single gradient in rate, decreasing basipetally, in early stages of echinoderm development, with a secondary gradient in the opposite direction appearing in the basal region as gastrulation approaches, or in the starfish somewhat later, and local gradients of arms and oral lobe appearing still later. It does not, however, give any information as to presence or absence of the concentration gradients of Runnström.

Fifth, the organization of the eggs of many organisms appears to be an incidental, rather than a fundamental, factor in development—a factor determined by the differentiation of the egg during its ovarian history, maturation, and fertilization. The ascidian egg, for example, evidently possesses a relatively high degree of orderly regional differentiation before the first cleavage begins; but an ascidian can develop from a bud at the tip of a stolon or elsewhere, from an isolated piece of ascidian body or stolon, and from the cell aggregates formed in many species within the bodies of degenerating individuals. There is no evidence of an organization similar to that of the egg in these various forms of development; but they may all give rise to ascidians like those from eggs. Evidently the fundamental factors of ascidian pattern originate in some way in all. Are those factors primarily anything more than gradients in rate of living? A bud is apparently a local activation decreasing from a center; there is no evidence at present that it is primarily anything more than that. In reconstitution from pieces of hydroids, planarians, and many annelids a new organization originates and determines more or less the making-over of the old. Buds and reconstitution of isolated pieces or of aggregates of dissociated cells bring us closer to the beginnings of developmental pattern than do eggs of most animals. We must look to these forms of development for the fundamental factors of developmental pattern. Only by investigation and comparison of all the different forms of development can we hope to distinguish the fundamental factors of pattern from those which are incidental to a particular form of development. Many eggs at the beginning of cleavage are actually well along in development; but in the bud and in reconstitution of a new whole individual from an isolated piece or a cell aggregate, development of pattern begins anew.

Some biologists apparently believe that metabolism is not a fundamental factor in development. For example, Shearer (1930, p. 266) says: "Morphological organization

has nothing to do with metabolism"; Parker (1929, p. 424), in criticizing the physiological gradient concept, makes the statement: "The metabolic activity of the organism is not a true formative process, but the result of such a process." Spemann (1938, pp. 321 ff.) seems to hold somewhat the same opinion; his discussion of gradients involves the mistaken assumption that, according to the gradient theory, there must be only quantitative metabolic differences in the amphibian egg at the beginning of embryonic development. In his recent book Weiss (1939, pp. 373-83) seems to hold that specifically different capacities for organization exist in different regions independently of metabolism. Granting that these capacities are present in many eggs, how did they arise except through metabolism? And, even if they are present in eggs, are they present in the early stages of buds, of pieces of *Tubularia* or *Corymorpha* stem, or in aggregates of dissociated *Corymorpha* cells? In isolated pieces of the planarian body, of various annelids, and of some ascidians development of a particular part has no definite or constant relation to a particular region of the original individual or to a pre-existing organization; but a new organization originates. Does it originate independently of metabolism? If we inhibit metabolism, it does not appear; and in many cases it is possible to initiate its development by purely quantitative external differentials which in one way or another determine gradients involving metabolism. It has been pointed out repeatedly that, even if these gradients are primarily quantitative, they probably do not remain so for any great length of time, though quantitative factors may continue to exist and be effective, even in many adult animals. How do the regional organizing capacities and the presumably specifically different metabolisms of hydranth, stem, and holdfast region in a reconstituting piece of *Corymorpha* originate, if not through metabolism? Since any level of a new individual may develop from any level of the stem, these capacities are certainly not localized preceding isolation of a piece. In the absence of a gradient involving metabolism they fail to appear; with establishment of a sufficient gradient at any stem-level or at either end of a piece they do appear. The arguments of Spemann and Weiss are based on the egg and embryonic development rather than on development in general, but other forms of development throw more light on the origins of developmental pattern than does embryonic development at present. Like many other embryologists, they maintain that organization or specific capacity for it is present, but they do not tell us how it originates. If organization consists in localized presence of specific substances, how can these substances originate and be localized except in the final analysis through metabolism? Probably no one now believes that they are all present in the primitive germ cell. It is difficult to believe, for example, that localized formation of specific substances in the dorsal inductor region of the amphibian egg and embryo can take place independently of metabolism. Without metabolism ovarian development of the oocyte and embryonic development cease. Even the differentiation of various organs and tissues does not persist if their metabolism is decreased below certain levels. How are electric and other physiological regional differences established and maintained except through metabolism? What is the possible nature of formative processes assumed to be independent of metabolism? In short, is there any more fundamental characteristic of living protoplasms than metabolism? At present, evidence of any such characteristic seems to be wholly lacking. It may be argued that structure of some sort is more fundamental, but structure without metabolism is not living protoplasm and accomplishes nothing that can be regarded as organismic development. Even if the organization of the egg originates in the orientation of elongate dipolar and symmetrical or asymmetrical protein

molecules, as Harrison (1937) and others have suggested, the question at once arises: To what do the molecules orient? Metabolism is going on continuously in the ovarian oöcyte and in the parent organism to which it is attached. Can the orientation possibly occur without relation to this metabolism? In this connection, also, it is highly significant that in reconstitution of a piece of *Corymorpha* or of a planarian the new organization follows certain changes in metabolism in the region concerned and without them does not develop. Doubtless changes in structure are also involved and affect metabolism, but metabolism is the active and effective factor. There is, at present, no evidence indicating that the changes in metabolism characteristic of the new organization result from orientation of protein molecules in all the thousands of cells concerned. The polarities of the individual cells of the hydroid and planarian are apparently determined by local surface-interior differences without direct relation to the axiate pattern of the whole, but these cells become parts of the axiate pattern of the new individual and still maintain their original polarities. If their molecules are, or become, oriented, are they oriented with respect to the local surface-interior factors or to the axiate pattern of the whole individual? If they are locally oriented, then the new pattern is independent of them. If the new pattern is determined by their reorientation, how are their original polarities maintained?

Weiss, in his book, has much to say of the field concept, of field energy, of strong and weak fields, and of decrease in field energy from a center; but he does not tell us what a field is or may be as an active and effective factor in development, nor does he say what makes a field strong or weak or what the source and character of its energy may be. What is the basis for decrease of field energy from a center to periphery of the field? New fields originate in the course of development; how do they arise? Is their origin independent of metabolism? Is there some other source of energy in a field than metabolism? Without information or at least hypothesis concerning these points, the field concept remains almost mystical in character. It does provide a formal designation for particular regions of a developing organism in which particular results appear, or may appear, under experimental conditions. Much has been learned from transplantations and other lines of experiment concerning extent and times of appearance of certain fields in certain organisms, but little concerning their nature. Until the field has content of some kind which can accomplish the observed results, the field concept remains at best a purely descriptive and formal summary of observation and experiment, or, as the term "field" is often used, little more than a verbalism, a symbol of something unknown. It does not advance our knowledge of the physiology of development. Is a field in its dynamic aspects primarily anything more than a gradient system of a particular kind of metabolism in a particular kind of protoplasm? Can it, in the light of all the lines of evidence available, be anything more than that? Organ fields evidently originate in relation to a more general pattern; and, proceeding from later to earlier stages, we finally come to the field, pattern, or gradient system of the individual.

That organization with localized specific differences of substance and metabolism does originate in some way and that dynamic, not merely structural, factors are essential to its origin appears beyond question; that regional specificities originate and increase during development is indicated by many lines of evidence. If metabolism is merely incidental to, or a result of these changes, it would seem that we must again postulate a specific vital energy; Driesch's entelechy will scarcely serve our purpose, for that was

conceived not as a source of energy but rather as controlling energy transformation—and that in living protoplasm is, in the final analysis, controlling metabolism.

The concept of physiological gradients in terms of dynamic factors effective in bringing about development, rather than of purely structural factors, does not in any way conflict with the concept of organization in terms of specific, regionally localized material differences. It merely maintains, on the basis of many lines of evidence, that such organization is not the primary pattern of development but the result of metabolic activity in a primarily quantitative pattern from which the regional specificities gradually developed. In short, this concept is an attempt to look beyond organization already present and to throw some light on the problem of its origin and development.

SUMMARY

1. Exposure to LiCl in the earliest stages of *Dendraster* development inhibits prospective ectoderm more than prospective entoderm. As gastrulation approaches, its inhibiting action on entoderm increases and becomes greater than on ectoderm. It may bring about partial or complete breakdown of entodermal epithelium into a solid cell mass or a dissociation into free cells.

2. All degrees of differential inhibition of ectodermal development result from lithium action: pluteus forms with arm angle decreased, more or less parallel or fused arms, single median arms, oral lobe partially or completely inhibited, complete inhibition of arm development, obliteration of ventrodorsality and even of polarity.

3. With sufficient concentration and exposure period lithium entodermizes prospective ectoderm progressively from basal levels acropetally, but it also inhibits further development of the entodermized ectoderm, as well as of the original prospective entoderm. Development of skeleton by mesenchyme is also decreased or completely inhibited.

4. Entodermization of ectoderm is apparently a result of inhibition or depression in the primary apicobasal gradient. It appears first in basal ectoderm because less inhibition is necessary to entodermize these than higher levels. In short, lithium is apparently a general and highly effective inhibitor of development. There is no evidence of specific regional effects of lithium on early development. Crowding produces similar differential inhibition and entodermization in *Dendraster*; and Janus green, in the starfish, *Patiria*.

5. The evidence indicates that exogastrulation results primarily from nonspecific differential inhibition rather than from specific regional effect of lithium or other agents, but secondary modifications of exogastrulae with great increase in size of entoderm may result from differential tolerance, conditioning, or recovery.

6. Two types of differential modification are commonly called exogastrulae. In true exogastrulae the original prospective entoderm evaginates instead of invaginating. In other, so-called exogastrulae the original entoderm may invaginate, break down into a solid cell mass, or undergo more or less complete dissociation into free cells. In these forms the external entoderm consists largely or wholly of entodermized ectoderm. Since entodermized ectoderm retains the same relations to other parts as when it was prospective ectoderm, questions whether it is inside out and which is its apical or anterior end arise and are discussed. It is suggested that the anterior end of the entodermized region may be the end connected with the remaining ectoderm, and it is pointed out that differential dye reduction supports this view. The small, thin-walled "neck," connecting remaining ectoderm and external entoderm in many forms with secondary modification

following the more extreme inhibitions, may perhaps be a stomodeum localized in the prospective anal region or a proctodeum. Development of the ciliated band in a circle about the flattened basal side of the remaining ectoderm after obliteration of ventrodorsality also suggests that a sufficient differential inhibition followed by recovery may bring this region into a condition somewhat resembling that of a ventral region.

7. Secondary modifications, opposite in direction from those of differential inhibition, result from differential tolerance or from conditioning (acclimation) to low lithium concentrations, and in more extreme degree from differential recovery after return to water, even from strongly inhibiting concentrations.

8. Secondary modifications after slight inhibition consist in relative enlargement of oral lobe and in increase in arm angle with short and small pluteus body. Excess of skeleton and supernumerary arms may also develop as secondary modifications, even in exogastrulae. The original prospective entoderm, inhibited to a solid cell mass and perhaps even after partial dissociation, is apparently able to regain, to some extent, its epithelial character during recovery. Entodermized ectoderm increases in size and may undergo further regional development, particularly in recovery. There is also evidence of re-ectodermization of the more apical levels of entodermized ectoderm.

9. In exogastrulae with entodermized ectoderm there is extensive reconstitution. The apical half or less than half of the original prospective ectoderm may develop arms or an arm and may approach pluteus form more or less, all parts except the extreme apical region developing from other than their original prospective regions. Entoderm of the more extreme exogastrulae may consist largely or wholly of entodermized ectoderm; but, if the original entoderm is not completely dissociated, it may form some part at the tip of the external entoderm, but much less than it would normally form and in harmonious development with entodermized ectoderm. In this case reconstitution involves production of less, instead of more, than normal. Entodermized ectoderm without original entoderm may develop three regions, separated by constrictions.

10. An appended review of the physiological gradient concept discusses questions of organization and its origin and points out that this concept does not deny existence of organization as an essential factor in development but is an attempt to throw some light on the problem of its origin.

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REGENERATIVE AND ACCESSORY GROWTHS IN PLANARIANS II. INITIATION OF THE DEVELOPMENT OF REGENERA- TIVE AND ACCESSORY GROWTHS¹

(Twelve figures)

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DURING the early part of the experimentation described in a previous paper (Goldsmith, 1939) animals with a supernumerary head in the mid-dorsal region were observed in stock cultures. At various other times individuals with one type of injury or another have been seen. Randolph (1897, p. 357) reported finding a specimen of *Planaria maculata* "with a cup-shaped growth on the back near the posterior end." It therefore seemed possible that in some cases the supernumerary structures had arisen from an injured region. A series of experiments was designed to test the ability of planarians to develop a supernumerary structure following an injury which does not completely separate two regions, and possibly by this means to add to our information on the phenomenon of induction.

In the vertebrates it has been shown that the action of the dorsal lip of the blastopore can be simulated by a considerable number of foreign tissues, both living and dead, and by specific chemical agents. It has also been demonstrated in a number of cases that nonspecific stimuli will evoke a definite structure, the nature of the structure depending upon the region which is stimulated.

For the invertebrates the following work should be noted. Browne (1909), using individuals of *Hydra viridis*,³ concluded that any part of the *Hydra* body except the tentacle region could give rise to a new hydranth if properly stimulated. The stimulus, however, can be given only by material lying at the base of the tentacles or by similar tissue in a regenerated hydranth or bud. Rand, Bovard, and Minnich (1926) reported that peristome tissue of *Hydra oligactis* (= *fusca*) exerted "a stimulating or positive form creating dominance over column tissue." Child produced supernumerary hydranths in *Corymorpha* by means of single and radial incisions (1927, 1929) and by implants of tissue taken from various levels of *Corymorpha* stems (1929, 1935).

In flatworms the following observations are of interest as applied to the production of supernumerary structures by local stimulation.

¹ Revision and extension of a dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy at Harvard University.

² I wish to express my thanks for the use of facilities in the departments of biology at Washington Square College, New York University, and at the City College of New York and for a grant from the Samuel Greenbaum Scholarship Fund of the City College of New York.

I take pleasure in acknowledging the helpful criticism and advice given throughout the course of this investigation by Professor H. W. Rand.

³ Hyman (1930) points out that *Chlorohydra viridissima* (Schulze) (= *Hydra viridissima* Pallas, *H. viridis* Linnaeus), and *Pelmatohydra oligactis* (Pallas) Schulze (= *Hydra oligactis* Pallas = *Hydra fusca* Linnaeus).

Randolph (1897) "punctured" ten planarians "in the side with a needle." Her results were negative, in that 22 days after the operation seven remained alive and were typical in appearance.

Gebhardt (1926) transplanted regenerating planarian tissues from various body regions and found that the fate of the grafted regenerate depended upon its age and the differentiation it had undergone prior to transplantation. Goetsch (1929) transplanted head regenerates into the postcephalic regions of other planarians. The portion of the host body anterior to the implant was removed. The graft reorganized the remaining host body into what resembled a postcephalic region.

Santos (1929, 1931) performed a series of homoplastic and heteroplastic transplantations in *P. maculata* and *Planaria dorotocephala*, each species serving (interchangeably) as host and donor. The cephalic ganglia, prepharyngeal, pharyngeal, and postpharyngeal regions were used as grafts and, with the exception of the ganglionic region, as sites of implantation. Santos concluded that a graft containing cephalic ganglia could act as an organizer or as a reorganizer, in that it may develop into a "head of some sort," which may bring about the reorganization of a region posterior to its level with the formation of a secondary pharynx and may completely reverse the polarity of a region anterior to its level. Miller (1938), using a modification of Santos' technique, successfully implanted cephalic grafts in the head, pharyngeal, and tail regions. Pieces of *Planaria gonocephala* containing cephalic ganglia and those from between the head and pharynx induced pharynges in the postpharyngeal regions of the host (Okada and Sugino, 1934, cited by Miller, 1938). Injection of planarian head extracts into the postpharyngeal region of individuals of *Euplanaria dorotocephala* produced no visible effect (Buchanan, 1938).

EXPERIMENTAL

Three different techniques were employed to ascertain whether a supernumerary structure could be produced in planarians by the application of a local stimulus: (1) radial incisions, (2) faradic stimulation, and (3) cautery. The animals were placed on a paraffin base, operated upon, and immediately returned to tap water. No anesthetic was used. Those cases in which the injury appeared to extend more than halfway through the parenchyma (dorsoventrally) and those in which the injury extended laterally so that complete discontinuity resulted (Figs. 1A and 1B) were discarded. In a number of successful cases in the cautery group only the superficial parenchyma was affected.

Since, of the three methods, the cautery procedure was found to give the greatest percentage of positive results, it was utilized to the greatest extent.

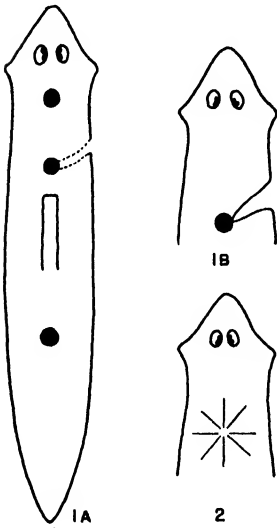


FIG. 1A.—The black areas indicate the regions to which the stimulus was applied. The broken line indicates lateral extension of the injury.

FIG. 1B.—Complete discontinuity resulting from lateral extension of the injury. Animals in which this occurred were discarded.

FIG. 2.—A series of "radial incisions."

RADIAL INCISIONS

Incisions approximately 0.5 mm. in length, radiating from a center, were made with a cataract knife in individuals of *Dugesia tigrina*⁴ and *Phagocata woodworthi* (Fig. 2). The incisions were made 1-3 mm. posterior to the eyes in animals ranging from 9 to 11 mm. in length.

Of about twenty-five specimens of *D. tigrina* so treated, several developed bulges at the site of injury after the wound region had closed completely. With the exception of two cases the elevated portion flattened out. One of the two developed into a small outgrowth, and the other into a small headlike structure containing what appeared to be typical eyes. The accessory head shifted its position by moving anteriorly so as to lie in close proximity to the original head.

Seventeen individuals of *Phagocata* were similarly treated. In six, pronounced bulges formed in the incised area after the wounds healed. One was resorbed, two developed into cylindrical outgrowths, one developed into an outgrowth with eyes, and two developed into outgrowths which finally became headlike and migrated anteriorly (Pl. I, Fig. 3).

FARADIC STIMULATION

A platinum electrode joined to an inductorium was touched to the dorsal surface of specimens of *D. tigrina*. The electrode was applied on the break, and the current was of an intensity sufficient to rupture the dorsal epithelium and to injure slightly the superficial parenchyma.

Twenty-five animals were so treated in the region 1-2 mm. posterior to the eyes, and twenty-one in the region posterior to the pharynx.

For the prepharyngeal series the following results were obtained: (1) one pronounced conical outgrowth which was resorbed after 10 days; (2) two slight bulges which developed into minute outgrowths and then pinched off at the base; and (3) one in which a single eye and another in which two small eyes developed at the site of injury.

In the postpharyngeal series one developed an elevation at the point where the electrode had been applied, and another formed a small outgrowth lateral to the site of the injury. The elevation subsided, and the outgrowth was resorbed.

CAUTERY

Bardeen (1901) "tried cutting planarians in two with a red-hot knife to see if cauterization would prevent the formation of tissue." The animals did not survive the operation.

The animals used in this group of experiments were *D. tigrina*, collected in the vicinity of Woods Hole and in Waverley, Massachusetts, and *Proctotyla fluviatilis*⁵ found in Waverley. The animals varied from 8 to 15 mm. in length.

A platinum electrocautery needle was heated slightly and was applied for a fraction of a second to various regions of the flatworm body (Goldsmith, 1932a, 1933). The greater number of experiments involved the dorsal aspect of the animal in prepharyngeal

⁴ In a private communication Dr. Libbie Hyman writes that the name *D. tigrina* (Girard) 1850 is the correct one. In previous papers I have used the name *P. maculata*. Other synonyms which occur in the literature are *Planaria lata*, *Euplanaria maculata*, *Euplanaria novangliae*, and *Euplanaria tigrina*. *Phagocata woodworthi* has previously been called *Phagocata gracilis* (Hyman, 1937).

⁵ *Proctotyla fluviatilis* has erroneously been called *Dendrocoelum lacteum*. *Dendrocoelum lacteum* is a European form and is not represented in America (Hyman, 1928).

and postpharyngeal regions. Some of the planarians were burned in a region slightly posterior to the eyes; others slightly anterior to the pharynx; and others, slightly posterior to the pharynx (Fig. 1A).

After some practice in the use of the cautery, it was found that the area injured could be fairly well localized. If, within several days, no bulge appeared at the site of injury, the region was recauterized. The injured surfaces measured 0.2–1.0 mm. in diameter, those of the latter dimension occurring least frequently. In the cases in which the burned region was not circular in outline, the area would also fall within these limits.

Of the fifty cases of *Procotyla* operated upon, no bulges or outgrowths were obtained. The lack of pigment in the animal makes it difficult to determine the extent of the injury and the amount of tissue regenerated.

In the remainder of the work, *D. tigrina*, to which the subsequent statements apply (except where otherwise indicated), was used.

TABLE 1
THE FATE OF STRUCTURES PRODUCED BY THE CAUTERY OPERATION

Region Cauterized	Number of Cases	Number of Elevations	Number of Elevations in Which Eyes Did Not Develop and Which Subsided	Number of Elevations in Which Eyes Developed and Which Subsided with Eyes Remaining	Number of Elevations Developing into Outgrowths	Number of Elevations Developing into Outgrowths with Eyelike Pigment	Number of Elevations Developing into Outgrowths with Eyes (Curved Pigment Masses in Clear Areas)
Prepharyngeal . . .	181	60	18	9	11	1	21
Postpharyngeal . .	169	54	36	0	13	3	2*

* In these two cases the anterior portion, including the pharyngeal region, of the animal was removed immediately after cautery.

RESULTS

The results obtained from similar injuries are not identical, and it was found that, in general, the tissue in the region anterior to the pharynx underwent further differentiation than did the tissue in the region posterior to the pharynx.

As a consequence of operations upon 181⁶ animals in the prepharyngeal zone and upon 169 animals in the postpharyngeal region the structures obtained may be classed as follows: (1) supernumerary eyes; (2) bulges which persist for a few days or even several months without further differentiation and which at the end of these periods of varying duration are entirely resorbed; (3) bulges in which an eye or eyes develop and which are resorbed with the eye or eyes remaining in the body tissue; and (4) bulges which develop into outgrowths which (a) may pinch off a distal portion, and the proximal portion may be resorbed or else may give rise to a new distal region, (b) may form an aborted head, (c) may slowly shift their position so that they come to lie more and more anteriorly, and finally appear as if they were one of the members of a double head of the "fused" head type resulting from the splitting operation previously described (Gold-

⁶ These were the cases observed for varying periods of time. Several hundred others, in which the injury was too deep or extended too far laterally, were discarded.

smith, 1939), or (*d*) may gradually become more definitely headlike but may persist in appearing as outgrowths from the dorsal surface of the animal. The data are summarized in Table 1.

In two cases structures which could be interpreted as supernumerary tails developed following the cautery operation in the postpharyngeal region. In one of the animals a

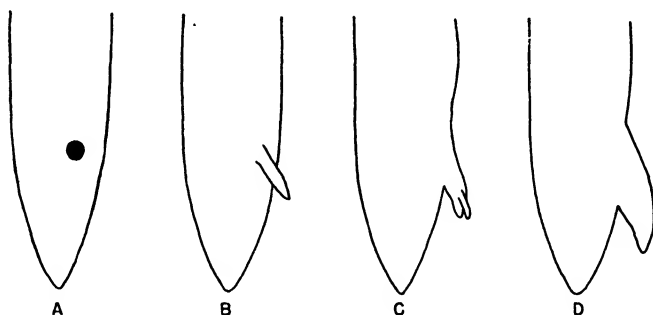


FIG. 4.—*A*, the black area indicates region to which cautery was applied. *B*, development of an outgrowth. *C* and *D*, the outgrowth diminishes in size and is finally resorbed. The original tissue extends laterally and posteriorly, so that a structure similar to a supernumerary tail produced by the splitting operation develops.

small indifferent outgrowth developed at the site of the injury. This was resorbed. In the other, no outgrowth developed. In both cases the original tissue lateral and posterior to the region injured extended laterally and posteriorly, so that a structure, similar to a supernumerary tail arising from splitting the posterior portion of a planarian to one side of the mid-line, was produced (Fig. 4).

FORM OF OUTGROWTH

The outgrowths in general are somewhat conical or cylindrical and may have from one to five eyes (Fig. 5). Where the eyes are more than two, they are usually distributed over the anterior, posterior, and lateral aspects of the growth. All surfaces are similar in pigmentation to the dorsal surface of the animal, from which the outgrowth arises.

Double outgrowths, such as those shown in Plate I, Figure 6, may develop. Outgrowths of this type appeared as if they were composed of two heads (each having a set of eyes), which had fused by their ventral surfaces. These structures usually developed at a site which had been cauterized more than once. It is possible that subsequent cautery operations involve not quite the same zone as the previous operations. If two active centers are set up, the "double" outgrowths may result.



FIG. 5.—Diagrammatic outline of an outgrowth with five eyes produced by cautery.

MIGRATION

The outgrowths exhibited the tendency to migrate anteriorly and laterally, so that a structure originally slightly anterior to the pharynx may finally come to lie between, or lateral to, the eyes of the host (Pl. I, Figs. 7, 8, and 9).

Santos (1931) observed this tendency in postcephalic grafts. The direction of movement here depended upon the orientation of the transplant, so that a graft in reversed anteroposterior orientation may migrate with its anterior end leading, moving farther and farther from its original body-level. Miller (1938) has also noted this migratory tendency of grafts.

Migration of grafts in *Hydra* have been reported by Rand (1899) and by Hefferan (1902).

RESORPTION

It has been observed that an elevation and even a small outgrowth arising as a result of a local stimulus may persist for a short time and then be gradually resorbed. The

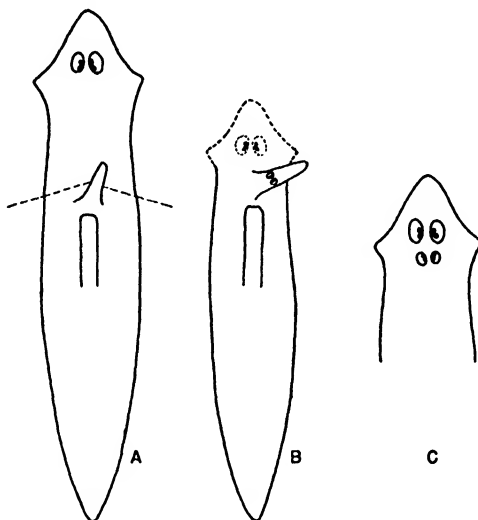


FIG. 10. —A, a conical outgrowth developed after cautery. The anterior portion of the body is removed (incision indicated by broken line). B, eyes developed in the outgrowth and a head is regenerated. C, the outgrowth has been resorbed, but its eyes remain.

resorption time varied greatly. In one case the induced outgrowth was small and was resorbed within 25 days after the cautery operation; in another, the outgrowth remained for 70 days; and in a third, resorption was complete after 4 months. In the animal shown in Figure 10 resorption did not begin until 11 months after the initial appearance of the outgrowth. A month later only the eyes remained (Fig. 10). In a similarly treated planarian the outgrowth gradually decreased in size but was still present 13 months after its origin. It should be pointed out that in many cases the outgrowths have remained and have maintained their size and activity (Pl. I, Fig. 11).

In connection with this resorption of outgrowths the utilization of a supernumerary eye in the formation of a typical head was observed. In this case a small mound of tissue developed after cautery of the region slightly posterior to the eyes (Fig. 12, A). Nine days after its appearance the mound subsided, and an eye which had developed in it remained. The original head was removed by a transverse cut posterior to the original

eyes (Fig. 12, *B*). Tissue which shaped up as a typical head regenerated anterior to the remaining eye (formerly the supernumerary one). Its vesicle increased in size, and an additional pigment cup developed in it. A new left eye was formed (Fig. 12, *C*).

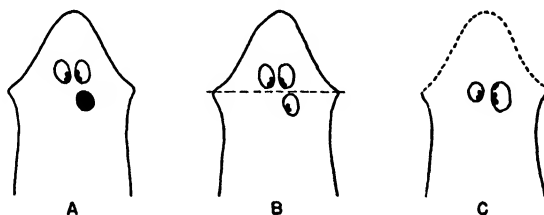


FIG. 12.—*A* and *B*, development of a supernumerary eye following cautery. The original head is removed, as indicated by broken line. *C*, a head with a left eye is regenerated. In the development of the right eye the supernumerary one is utilized.

DISCUSSION

The observations recorded in this paper demonstrate the ability of several types of injury to evoke a response which may culminate in a headlike structure. They further indicate that the degree of differentiation which the structure may undergo is, in part, dependent upon the region in which the operation is performed. It was found that structures in prepharyngeal regions developed headlike morphological characteristics which, with two exceptions,⁷ did not obtain for those in the postpharyngeal area. Since no foreign tissue was involved, it can be definitely stated that the tissue of the injured animal has differentiated to form the new growth.

Gebhardt (1926), Santos (1929, 1931), and Miller (1938), by grafting pieces of planarians, produced outgrowths very similar in appearance to those described in this paper. Santos concluded that the ganglionic graft is an organizer in the sense of Spemann. In its new location the graft develops into a headlike structure and then induces an outgrowth from the host body (part of the outgrowth appears to rise from donor tissue). The sequence of events in my experiments is quite different. The injured region becomes elevated. This elevation increases in size, so that it protrudes markedly; and eyes and auricles may develop in the distal portion of the structure so formed.

A similar phenomenon is recorded by Child (1927) for *Corymorpha*, where "radial incisions" served to produce a secondary hydranth.

The behavior in the injured planarians and of the *Corymorpha* individuals described by Child would then necessitate shifting the emphasis from the agent utilized (injury or implantation of tissue) to the zone to which the agent is applied or transplanted. This concept is in harmony with the facts of induction derived from the work on *Amphibia*.

Supernumerary limbs, composed of host tissue, developed following the implantation of otic vesicles (Balinski, 1927*a*), nasal placodes (Glick, 1931), tailbud (Goldsmith, 1932*b*), and celloidin (Balinski, 1927*b*). Implantation of foreign tissue or irritation of the middorsal line region of *Triton* induced a supernumerary crest of host tissue (Milo-

⁷ Immediately after cautery the entire anterior portion, including the pharyngeal region, of the animal was removed.

jevic, Grbic, and Vlatovic, 1926). The experiment of Guyénot and Schotté (1927), in which a regenerating limb bud with incipient digits formed "a small characteristic tail" when transferred to the side of a *Triton* tail, and Gebhardt's work with planarian regenerates further impresses one with the importance of the so-called "morphogenetic field."

Is the difference in the behavior of the prepharyngeal and postpharyngeal regions following radial incisions, faradic stimulation, and cautery in flatworms to be attributed to differences of the fields? The answer to this may be approached by roughly plotting the sequence of events after injury.

A region is injured. It is repaired by the addition of new tissue. The new tissue may do more than replace the portion lost or injured; it may continue to grow. There is now present something akin to the regenerates used as transplants by Gebhardt, but, differing in that, it is an "indifferent" regenerate, since it has formed at neither the head nor the tail end of a worm. Now if the injured region is a prepharyngeal one, the outgrowth may develop eyes and auricles; if it is the postpharyngeal region which has been irritated, the outgrowth will usually continue to be indifferent. This is shown by the data in Table 1.

Here we are dealing with the "host" alone. There is no donor tissue. It is realized that tissues, such as those of the nervous and digestive systems, will be exposed at the point of injury. In a review of the literature (Goldsmith, 1939) it was shown that at present we cannot regard the digestive influence as a formative influence in head regeneration. The influence of the nervous system in this connection is still not clear. Beyer and Child (1930) conclude that the nervous system may be a localizing influence but is not a determining factor in the formation of a head. Goldsmith (1939) found that in many cases head regeneration failed to take place although the lateral nerves were present. Further, it must be remembered that the exposure of the tissues occurs in both the prepharyngeal and the postpharyngeal regions. In view of the foregoing, it might appear that the potentialities of the two territories differ greatly, the prepharyngeal being able to form a head and the postpharyngeal being unable to do so. However, since a decapitated prepharyngeal piece and a piece from which the entire pharyngeal region has been removed will behave similarly in that both will regenerate heads, we must concede that such cannot be the case. That there is some difference between these regions cannot be denied, but the difference must be one of degree rather than of kind. That this latter view may approach the correct interpretation is in part borne out by cytological studies.

FORMATIVE CELLS

"Bildungszellen," regarded as the precursors of regenerating organs, were described in the parenchyma of rhabdocoels by von Wagner (1890). Keller (1894) stated that the number of these cells varied directly with the regenerative power of the species.

In individuals of *P. maculata* dividing by fission the number of formative cells is greatly increased in the region of the body dorsal to the gut, so that they become the most numerous of the mesodermal constituents (Curtis, 1902). Additional work (Weigand, 1930; Curtis and Schulze, 1934) has led Curtis (1936) to regard the formative cells in planarians as those functioning in the formation of new parts during regeneration. However, "the immediate origin of these cells is not so obvious." Child and Watanabe (1935) are of the opinion that, following removal of an anterior region, partially differentiated cells (not undifferentiated formative cells), owing to a change in their "physio-

logical environment," become embryonic and may then give rise to a new region. Curtis and his collaborators have made a comparative study of the number and distribution of the formative cells in normal and irradiated individuals of *P. maculata*, *Planaria agilis*, and *Procotyla fluviatilis*. The number of formative cells in the former two which exhibit marked powers of regeneration is considerably higher than in *Procotyla*, which has a low regenerative capacity (Lillie, 1901; Morgan, 1904; Isely, 1925). Cell counts made in individuals of *P. maculata* from a stock in active fission show that the number of formative cells mounts rapidly, beginning at a point slightly posterior to the eyes, reaching a maximum at a point midway between the eyes and the anterior extremity of the pharynx. The number then decreases gradually until the pharyngeal region is reached, where an abrupt diminution is observable. The number then remains low, until the level of the second zoöid is reached. Here there is a second increase, followed by a sharp decline in posterior tail region. *Procotyla*, like *P. maculata*, contains more formative cells in its prepharyngeal region than in the postpharyngeal, but the absolute number is lower (Curtis and Schulze, 1934).

Table 1 shows clearly that the differentiation attained by anterior structures is greater than that attained by posterior ones (two exceptions; in these the entire anterior region, including the pharyngeal portion, had been removed at the time of cautery). The outgrowths arising in the regions corresponding to those said to be highest in formative cell content (Curtis and Schulze, 1934) are those in which eyes develop and which in some cases take on a headlike appearance. It was in this zone that the incidence of head regeneration was highest following removal of one head in a double-headed planarian (Goldsmith, 1939).

In a number of instances where the cautery operation was followed by wound healing but no outgrowths, repetition of the operation gave positive results; in one case an outgrowth developed only after the sixth cautery operation.

Flexner (1898) noticed that, following transection in *Planaria torva*, there is a marked production of new cells. These Curtis (1902) concludes are dividing formative cells. If these observations and their interpretation are correct, then by successive injuries one should be able to increase the number of formative cells per unit volume. This the successive cautery operations may have done, and consequently an outgrowth was formed.

From this it follows that every point along the anteroposterior axis of individuals of *D. tigrina* should respond similarly, were the stimuli sufficient in number and the intensity of the stimuli sufficient in degree.

The negative results following the cautery operation in fifty individuals of *P. fluviatilis* may be related to the smaller number of formative cells in *Procotyla*. Curtis and Schulze (1934) give the approximate ratio as "... 8.5 formative cells in *P. maculata* to 1 in *Procotyla fluviatilis* for equal unit areas in similar regions of the two worms."

If there is a relationship between the number of formative cells and the degree of differentiation which structures may undergo, can the nature of the relationship be defined in chemical terms?

Sulphydryl compounds.—The glutathione content in *Procotyla* is low, while that of *P. agilis* is high; further, the glutathione content is higher in regenerating tissues. These facts Coldwater (1933) correlated with the "formative cell" count. Coldwater, while showing that -SH compounds accelerated the rate of regeneration of posterior segments of *Tubifex*, was unable to produce a similar effect in *P. maculata* or *Procotyla*.

Recently, Owen, Weiss, and Prince (1938) found that glutathione (but not cysteine) stimulated regeneration in sectioned planarians and the fission-rate in whole animals. Similar effects were observed after treatment with 1:2:5:6-dibenzanthracene. Reimann and Hammett (1935) believe that the stimulating action of this, as well as of other compounds, may be exerted through the -SH group.

In a previous report (Goldsmith, 1934) the belief was expressed that the so-called "inductions" produced by various agents arose as a result of irritation. If this were true, then cautery, as used in the flatworms, should also act as an "evocator" when used on other organisms and should awaken potencies lying dormant. This has been confirmed by Cohen (1938), who induced various structures in *Rana pipiens* by inserting a microcautery needle into the blastocoel of the early gastrula. It was also pointed out (Goldsmith, 1934) that injury may release sulfhydryl⁸ (scratching a growing root tip resulted instantly in a freeing of sulfhydryl [Hammett, 1929]), which may accelerate cell division in those cells which are the relatively undifferentiated ones, the formative cells in planarians. Not only do the formative cells proliferate following an injury, but they differentiate; and the height of differentiation in planarians is expressed in head formation. It must be remembered that there is an inhibitory influence emanating from the anterior end of the animal (cf. Child, 1929; Goldsmith, 1939; and Weiss, 1939, pp. 380-82). The degree of differentiation which the developing structure will undergo will depend upon the ratio of the regenerative capacity of the injured region to the inhibitory action exerted by the apical end of the worm.

SUMMARY

1. Supernumerary eyes or small outgrowths with or without eyes may arise in individuals of *D. tigrina* (syn. *P. maculata*) following faradic stimulation.
2. Supernumerary structures, which in some instances develop into heads, may arise as a result of radial incisions (*Phagocata* and *D. tigrina*) or cautery (*D. tigrina*). In most cases the structures arising after injury to the prepharyngeal region differentiate to a greater extent than do those arising after injury to the postpharyngeal region. The similarity between these outgrowths and those produced by the implantation of planarian tissues is pointed out.
3. Repeated applications of the cautery needle may result in the development of a supernumerary outgrowth after previous applications (five in one case) had been unproductive.
4. In a number of cases the outgrowths migrated anteriorly and laterally, so that a structure originally slightly anterior to the pharynx may come to lie between or lateral to the eyes of the "host."
5. The outgrowths may persist for variable periods of time and then be gradually resorbed. Eyes which had developed in these structures remain as supernumerary eyes in the "host" tissue long after the induced growths have been resorbed.
6. Individuals of *Procotyla* reacted negatively to all the stimuli.
7. It is suggested that the high degree of differentiation attained by the outgrowths induced in the prepharyngeal region in *Dugesia* and the negative results in *Procotyla* may be correlated with the "formative cell" distribution. These in turn may be related to the presence or release of sulfhydryl.

⁸ Although a mass of data in support of the formative cell -SH concept is available, the evidence is not entirely in its favor; and we must be careful, in the present state of knowledge, not to regard the -SH group as omnipotent.

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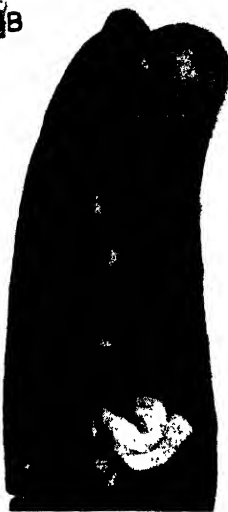
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PLATE I

2A



3B



4



7A



7B



8



9A



9B



11



PLATE I

FIG. 3*A*.—A dorsal view of an individual of *Phagocata* showing the supernumerary head (to the right of the original head) which developed following "radial incisions."

FIG. 3*B*.—A ventral view of the animal shown in Fig. 3*A*.

FIG. 6.—An outgrowth possessing two sets of eyes, one pair on the anterior face and another on the posterior face. The pigment cups in the clear areas (vesicles) do not show in the photograph. An additional structure arises from the left side of the outgrowth.

FIG. 7*A*.—A dorsal view showing a supernumerary head which had migrated from the mid-dorsal, prepharyngeal region.

FIG. 7*B*.—A ventral view of the animal shown in Fig. 7*A*. It is clear that the supernumerary head arises from the dorsal side.

FIG. 8.—A supernumerary head lies directly posterior to the eyes of the original head. Many of the outgrowths migrate anteriorly, as did this one.

FIGS. 9*A* and 9*B*.—A dorsal and ventral view of a supernumerary head which had migrated laterally.

FIG. 11.—An outgrowth (15 months after its initial appearance) arising from the dorsal aspect of the animal. One vesicle is visible. The pigment cup in it cannot be seen. Another eye is present on the other side of the outgrowth.

The supernumerary structures shown in Fig. 6–11 were produced by cautery.

SOMATIC EFFECTS OF TEMPERATURE ON DEVELOPMENT IN *DROSOPHILA MELANOGASTER*. I. PHENOCOPIES AND REVERSAL OF DOMINANCE

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MANY investigators of the effects of high temperature on various stocks of *Drosophila melanogaster*—Goldschmidt (1929, 1935), Jollos (1930, 1933), Plough and Ives (1932, 1935), and others—have noted among the imagoes heated in prepupal stages individuals which phenotypically resembled genotypes other than the ones used. These "phenocopies" (Goldschmidt's term) included stubby, forked, extra, and missing bristles; rough, and deformed eyes; straplike, balloon, spread wings; certain eye and body colors; and many other characters. In short, representatives of nearly every type of mutant character were obtained. The data, particularly those of Goldschmidt, indicated that the extent and type of the effect were dependent on four factors: (1) the temperature used, (2) the duration of the treatment, (3) the genetic constitution of the treated flies, and (4) the developmental age of the flies at the time of treatment.

In 1935 a series of investigations was started in the Amherst laboratory to determine more precisely the various factors involved, particularly the importance of the genetic constitution and the age of the flies at the time of treatment. An inbred wild stock, Florida +10, and F_1 heterozygotes of this stock against multiple mutant strains representing the first, second, and third chromosomes were used in these experiments. It was expected that the heterozygotes would be more sensitive than the wild-type flies to the effects of temperature. At the same time, effects might be visible which would be masked by the still greater effects of genes in homozygous condition.

In these experiments the heterozygotes and the wild-type flies were subjected to a 12-hour exposure to 36.5 ± 0.2 C. at definite periods during larval and pupal development. The control temperature was held at 25.3 ± 0.2 C. Egg-laying periods were from 2 to 6 hours in duration.

The phenocopies obtained in these experiments represent a wide range of types. The characters most frequently found include: alterations in size and form of eyes, legs, wings, bristles, and abdomen; presence or absence of specific wing veins and body bristles; and modifications in eye and body color. These and other phenotypes provide numerous close parallels to the known series of mutant characters involving such effects. Thus, the phenotypes appearing after temperature change during development are often similar to those produced by mutant genes acting when the temperature is constant; or, stated in another way, an individual of one genetic constitution raised at one temperature resembles phenotypically an individual of a different genetic constitution raised at another temperature.

The data included in Tables 1-4 were selected to demonstrate the phenotypic expression of the nominally recessive genotypes of the treated stocks. The results indicate the percentage of flies showing the particular character produced following heat treat-

ment at a specific time in development. The incidence of two or more phenotypic characters in the same fly is not tabulated, since a significant correlation between such characters was indicated in only one case, i.e., the connection between truncate and curved mentioned below.

OBSERVATIONS

As Goldschmidt (1935) and others have already observed, the time at which certain phenocopies may be induced is very specific. Inspection of the data in Tables 1-4 shows that the temperature-effective periods for different characters are not of the same duration and may occur at different periods in development. For example, eye shape seems

TABLE 1
PHENOTYPIC EFFECTS ON WILD-TYPE STOCK (FLORIDA +10) OF EXPOSURE
FOR 12 HOURS TO 36°5 C.
(Data calculated in percentages)

PHENOTYPES OBSERVED	AGE IN DAYS AT TIME OF TREATMENT							
	Control	3.0	3.5	4.0	4.5	4.75	5.0	5.5
Rough eye.....	0 4	10.0	45.4	60.0	10.0	25.6	1.8	3.1
Short wing.....	0	4.0	9.1	13.3	2.0	3.8	0	16.7
Curved wing.....	0	0	0	0	0	2.6	0	15.0
Truncate wing.....	0	0	0	0	0	1.3	0	0
Vortex.....	0	0	0	0	0	0	0	0
Plexus.....	0	0	0	0	0	0	0	0
Crossveinless.....	0	0	0	0	0	0	0	0
Forked bristles.....	0	0	0	0	0	0	0	32.9
Short bristles.....	0	0	4.5	3.3	2.0	10.3	10.9	16.7
Missing bristles.....	0	0	0	10.0	4.0	15.4	12.7	3.1
Microchaetes gone.....	0	0	0	3.3	20.0	0	3.6	10.9
Hairy thorax.....	0	0	0	0	4.0	0	0	0
Dark body.....	0	0	0	0	2.0	0	0	0
Total number of flies observed.....	235	50	22	30	50	78	55	64

to be thermally sensitive during most of the larval life-history, whereas certain wing and bristle characters respond to temperature treatment only at quite specific periods in early pupal life. Furthermore, the time of appearance of many phenocopies agrees closely with the facts already known about the ontogeny of *Drosophila*. Since the time during which puparium formation takes place at 25°3 C. was found to be from 4 to 5 days for +10 and +10/2ple flies, in the subsequent analysis 4.5 days is used as the time of puparium formation.

Among the phenocopies most specific in time of production are those affecting the appearance of the wings. Most of the wing characters were induced by treatment after the time of puparium formation. The data indicate that the sensitive period for truncate begins approximately at the start of actual pupation. The vein effects have also been localized largely in the first 12 hours after pupation. This conforms with the data of Hersh and Ward (1932) and Stanley (1935) on the temperature-effective period for

TABLE 2

PHENOTYPIC EFFECTS ON $+/1$ PLE (SCUTE, CROSSVEINLESS, VERMILION, FORKED)
OF EXPOSURE FOR 12 HOURS TO $36^{\circ}5$ C.

(Data calculated in percentages)

PHENOTYPES OBSERVED	AGE IN DAYS AT TIME OF TREATMENT							
	Control	3.0	3.5	4.0	4.5	4.75	5.0	5.5
Rough eye.....	0.5	7 7	64.3	77 0	18.6	22.2	0	0
Short wing.....	0	0	43.0	0	0	0	0	16.7
Curved wing.....	0	0	0	0	0	0	0	0
Truncate wing.....	0	0	0	0	0	0	0	0
Vortex.....	0	0	0	0	0	0	0	0
Plexus.....	0	0	0	7.7	14 0	11 1	32.0	0
Crossveinless.....	0	0	0	0	7.0	1.8	8 0	0
Forked bristles.....	0	0	0	0	9.3	3.6	0	37.5
Short bristles.....	0	0	0	15 4	7.0	1.8	20 0	31.2
Missing bristles.....	0	0	50.0	46.0	18.6	31.4	28.0	8.3
Microchaetes gone.....	0	0	0	0	18.6	7 4	20.0	2.1
Hairy thorax.....	0	0	0	0	0	0	0	0
Dark body.....	0	0	7 1	0	0	0	0	0
Total number of flies observed.....	216	13	14	13	43	54	25	48

TABLE 3

PHENOTYPIC EFFECTS ON $+/2$ PLE (DUMPY, BLACK, PURPLE, CURVED, PLEXUS, SPECK)
OF EXPOSURE FOR 12 HOURS TO $36^{\circ}5$ C.

(Data calculated in percentages)

PHENOTYPES OBSERVED	AGE IN DAYS AT TIME OF TREATMENT							
	Control	3.0	3.5	4.0	4.5	4.75	5.0	5.5
Rough eye.....	0 9	32.0	57 6	36 6	6 4	15.8	27 2	44.4
Short wing.....	0	4.4	11.5	3.8	2.1	1.6	1.6	6.2
Curved wing.....	0	0	0	0	2.1	3.2	1.6	3.7
Pointed wing.....	0	0	0	0	0	0	9.3	14.6
Truncate wing.....	0	0	0	0	1.1	1.6	23.4	23.4
Vortex.....	0	0	0	0	0	0	1.3	2.5
Plexus.....	0	0	0	0	16.0	3.2	37.6	42.0
Crossveinless.....	0	0	0	0	2.1	0	2.6	3.7
Forked bristles.....	0	0	0	0	13.8	0	7.8	9.9
Short bristles.....	0	0	0	5.1	2.1	0	0	4.9
Missing bristles.....	0.2	4.4	11.5	25.4	1.1	9.5	4.9	5.0
Microchaetes gone.....	0	1.5	0	0	28.7	4.8	28.9	33.3
Hairy thorax.....	0	0	0	0	0	0	0.7	1.2
Dark body.....	0.7	8 7	0	13.9	22.4	6.4	2.6	3.7
Total number of flies observed.....	439	69	26	79	94	63	308	81

normal wings and with the period during which wing phenotypes were found by Goldschmidt, Jollos, and Plough and Ives. The embryological evidence also fits in. Although Auerbach (1936) has localized the period of growing-out of the wing pouch in the mature larva and prepupa, the morphogenetic effect of the mutant dumpy, i.e., truncate wing, first becomes visible from 18 to 36 hours of pupal age (Auerbach, 1936; Goldschmidt, 1937).

Short wing also seems to have a temperature-effective period at 3.5-4.0 days. Auerbach has found that the mesothoracic buds divide into the separate thoracic and wing disks at this time, i.e., in the early part of the third instar. She believes that vestigial, whose temperature-effective period begins at this time, acts during the third instar by effecting a reduction in the size of the wing disk. A similar explanation may account for the early sensitive period for short wings.

TABLE 4

PHENOTYPIC EFFECTS ON $+/3$ PLE (ROUGHOID, HAIRY, SCARLET, PEACH, SPINELESS SOOTY) OF EXPOSURE FOR 12 HOURS TO $36^{\circ}5$ C.

(Data calculated in percentages)

PHENOTYPES OBSERVED	AGE IN DAYS AT TIME OF TREATMENT							
	Control	3.0	3.5	4.0	4.5	4.75	5.0	5.5
Rough eye	0 6	94 4	90 0	83 4	7 9	8.4	13.6	7 0
Short wing	0	1 9	22.5	33 3	0	5 0	0	21.9
Curved wing	0	0	0	0	0	0	1.2	0
Truncate wing	0	0	0	0	0	0	0	0
Vortex	0	0	0	0	0	0	0	0
Plexus	0	0	0	0	0	0	0	0
Crossveinless	0	0	0	0	0	0 8	0	0
Forked bristles	0	0	0	0	0	0	0	14.1
Short bristles	0	0	2 5	33.3	1.3	8.4	43.2	26.3
Missing bristles	0	0	12 5	16.6	10.5	12.3	17.3	0
Microchaetes gone	0	0	0	0	14.5	1.6	7.3	7.0
Hairy thorax	0	0	0	0	3.9	0	0	0
Dark body	0.3	24.1	25.0	16.6	30.2	8 4	17.3	17.6
Total number of flies observed	318	54	40	6	76	121	81	114

The sensitive period for rough and abnormal eyes seems to extend throughout the period studied. The period of greatest effect, however, appears to lie in the third instar, before the formation of the ommatidia, which takes place at the end of the larval period (Krafka, 1924; Chen, 1929; Medvedev, 1935).

The bristle and hair effects occur mainly in the postlarval groups. However, the sensitive period for extra bristles lies in the 3.5-4.0-day interval, while the temperature-effective period for missing bristles apparently extends throughout the observed periods with a peak at 4.0 days and a lesser one at 5.0 days. The prepupal effect on missing bristles agrees with the data of Child (1935) and of Ives (1939) on the temperature-effective period for scute. The bristle and hair effects, considered *in toto*, correspond in point of time with the evidence of Plunkett (1926) and Goldschmidt (1935). Robertson

(1936) has found that the trichogenic cells do not develop until 27 hours, and the bristles and hairs not until 30 hours after puparium formation.

Abnormal legs, most of which were characterized by crippled tarsae of the third pair, occurred largely in the 3.5- and 4.0-day period. These effects, resembling some phenotypes of crippled (Komai, 1926) were induced, as were those of Hoge (1915), by temperature before the morphogenesis of the legs, which takes place in the first 4 hours after puparium formation (Auerbach, 1936; Robertson, 1936).

INTERPRETATIONS

The occurrence of these phenocopies may be interpreted in terms of the rate theory of development. According to the current rate theory, the characters of an organism are determined by the rates and interactions of developmental processes, the rates and durations of which are modified by the interaction of genetic and environmental factors during development.

Variations in the developmental environment of the wild genotype produce only slight changes in the wild phenotype. Wild-type flies raised within the range of normal temperature show slight differences in facet number (Margolis and Robertson, 1937), wing size (Stanley, 1931, 1935; Hersh and Ward, 1932; Riedel, 1934), and other characters. At the extremes in the temperature range these differences are more accentuated. This suggests that the temperature coefficients of all the developmental processes in the wild type are not equal. The temperature coefficients, however, harmonize sufficiently to produce a fairly uniform phenotype. This comparative constancy has been explained by Plunkett (1932) as due to a natural selection of genotypes which will produce uniform phenotypes under ordinary conditions, i.e., within the normal range of temperature, humidity, and other environmental factors affecting the development of organism.

Variations in the developmental environment of a mutant genotype, however, produce marked changes in the mutant phenotype. The extent of the effect varies with each mutant genotype (Bar, vestigial, scute, and Dichaete series of alleles, abnormal abdomen, extra legs, etc.) and also with the type and extent of the environmental change (temperature, humidity, and food). With respect to the temperature effect, it may be supposed that the temperature coefficients of certain reactions, affected by the mutant gene or its products, differ markedly from the temperature coefficients of development as a whole. This differential effect on the rates of developmental processes produces different effects with variations in temperature. Varying food conditions, humidity, and other factors affect differentially the time of development (as a whole) and the duration of processes affected by the mutant gene, thus producing recognizable phenotypic differences.

A mutant, moreover, may be considered as a wild genotype with a single gene substitution. This genic variation produces an effect like a change in the external environment. The extent of its effect may be supposed to depend upon (1) the rates and interactions of the developmental processes affected by the mutant gene as compared to the effect of the wild-type gene on these processes; (2) possible effects of the mutant gene on reactions not affected by its wild-type allele (and the reciprocal of this); and (3) the temperature coefficients of the reactions affected by the mutant gene as compared to the wild-type gene. When considering the action of genes in development, then, the factors involved are rates and temperatures coefficients of developmental processes. Variations

in genotype may be supposed to be similar to variations in environmental conditions in so far as both affect the rates of developmental processes.

On the basis of this reasoning, short exposures of the wild type to high temperatures outside the range of the "normal" environment, but not long enough to kill the organism, should have a differential effect on the reactions going on at that time. The developmental processes may be expected to be thrown out of step with one another because of their different temperature coefficients. The phenotypic effect of this exposure, as mentioned previously, depends upon at least four variable factors: (1) the genotype, i.e., the rates and temperature coefficients of the reactions affected; (2) the intensity of the treatment, i.e., the temperature used; (3) the duration of the treatment; (4) the time of treatment, i.e., the reactions affected. The end-products produced by these reactions may differ quantitatively or qualitatively from end-products produced under normal conditions. These end-products may be the adult character, or they may be substances which will affect the reactions occurring at a later period in development.

It then follows that exposures of the wild type at certain periods may be expected to produce phenocopies. The results of these experiments show further that an effect on a character may be produced before its morphological ontogenetic processes appear; e.g., bristle effects may be produced by treatment during the early larval period (Tables 1-4), although the bristles are not laid down until more than a day after puparium formation. Ives (1939) has shown that similar effects may even be produced by treatment during the embryonic period. This may be interpreted as a temperature effect on chemico-embryological processes, which in turn determine the rates of visible reactions ensuing at a later period in development. This significant concept, illustrated by Wright (1934) and Powsner (1935), appears to have been overlooked by many workers who emphasize the latter type of effect, viz., on the visible processes. Other effects, e.g., the truncate wings, appear to be specific for the morphological developmental period of the organ.

Reversal of dominance.—As stated before, the data included in Tables 1-4 were selected to demonstrate a particular aspect of the production of phenocopies, i.e., the phenotypic expression of the nominally recessive genotypes of the treated stocks. This phenomenon is best described as a reversal of dominance. Although rough eye occurs in all the stocks, its percentage of manifestation is the greatest in heterozygous 3ple, which includes roughoid (ru). Shortening of the wing may be an effect of the second chromosome gene, dumpy (dp). When treated in the postlarval temperature-sensitive period, short wing is more than twice as frequent in $+/2ple$ as in the other stocks; fully half of the flies in the former group show the phenotype. After treatment in the larval period, the manifestation of this character is greatest in $+/1ple$ and $+/3ple$. This suggests that there is another critical period independent of that acted on by the dumpy gene.

Except for some cases in $+10$ stock, curved-wing phenotype is manifested only in $+/2ple$, which includes curved wing (c). It should be added that curvature of the wing is often associated with extreme dumpy. Truncated wing is practically unique in heterozygous 2ple stock. Pointed wing, which refers to an oblique flattening of the inner distal end of the wing, seems to be a slight truncate effect. Vortex, a pleiotropic effect of dumpy, also occurs only in the presence of that gene. Plexus, the presence of extra bits of vein in the distal wing cells, is of variable occurrence both as to position and extent. The proportion of $+/2ple$ flies which shows this character is high (42 per cent), as might be expected from the presence of plexus (px); but a smaller percentage (32 per cent)

of $+/1ple$ flies also shows the character. A partial loss of the posterior crossvein was observed in $+/1ple$ (8 per cent), which includes crossveinless (*cv*), and to a lesser degree in $+/2ple$ (3.7 per cent). The fact that plexus and crossveinless appear in the presence of both *cv* and *px* suggests a close interrelation between the action of these genes.

The bristle effects do not lend themselves so readily to analysis. The percentage of forked bristles in $+/1ple$ (37.5 per cent), which includes forked (*f*), is almost equaled in $+10$ (32.9 per cent) and is exceeded in $+/2ple$ (55.1 per cent). Short bristles, an effect of spineless (*ss*), are most frequent in $+/3ple$ (43.2 per cent), followed by $+/1ple$ (31.2 per cent). Missing bristles, characteristic of scute (*sc*), are found primarily in $+/1ple$ (50). The percentage of missing microchaetes seems to bear no relation to the presence of bristle-removing genes.

Dark body occurs in both $+/2ple$ (22.4 per cent) and $+/3ple$ (30.2 per cent). Trident is included with dark body in the data. The greater effect of sooty in chromosome 3, as compared with black in chromosome 2, is paralleled by the higher percentage of manifestation in $+/3ple$.

In all these cases the effect was variable in extent, suggesting different degrees of reversal of dominance. Crossveinless varied from a slight deficiency to an almost complete lack of the crossveins. A truncate series was obtained with all degrees from normal through a phenotype resembling that of the oblique allele to an effect as great as that of homozygous dumpy raised at 25° C. Vortex varied from slight whorling of microchaetes to distinct humps on the thorax.

DISCUSSION

From the foregoing it is apparent that a gene normally recessive, such as dumpy or plexus, may behave as a dominant under certain other environmental conditions. The degree of dominance of a gene is, therefore, a function of the environment within which the developmental processes take place. This concept is a corollary of the rate theory.

In the developmental processes leading to character expression both alleles of a gene (if both are present) affect the character. This has been well established for the color genes of rodents (Wright, 1927; and others), the vestigial alleles of *Drosophila* (Mohr, 1932; Goldschmidt, 1935; Harnly, 1936), the scute series (Serebrovsky, 1930; Dubinin, 1932; Child, 1936), and for many other genes and characters in plants and animals. It is self-evident for two mutant alleles, e.g., scute 1/scute 5, vg^{nw}/vg^{ni} , etc. It is not as apparent in the case of scute/wild type or *vg*/wild type, etc. The greater effect of the wild-type gene on the developmental processes hides the effect of the mutant gene, giving rise to the phenomenon of dominance. That the recessive gene does produce an effect, however, has been shown by Riedel (1934) for vestigial/wild.

On this basis the heterozygote and homozygous wild type should behave differently with changes in the external environment (Lutz, 1913; Hersh and Ward, 1932; Riedel, 1934; Stanley, 1935; Margolis, 1935). The effect of very high temperature should be even more striking because of the greater differences in the rates of the developmental processes at these temperatures. Normal dominance relations, dependent upon "normal" environmental development, should be upset by heat treatment at particular periods in development. These periods should, furthermore, coincide with the periods at which the homozygous wild type is affected (since presumably the same developmental reactions are being affected). The data presented show that this has been realized in these experiments. "Dominance has been reversed" for a number of genes.

SUMMARY

1. Phenocopies representing most of the known types of mutant characters in *Drosophila* have been obtained by subjecting wild stock and F_1 heterozygotes of this stock against multiple mutant strains representing the first, second, and third chromosomes to 12-hour exposures to 36.5°C . at specific periods in development.
2. End-results of temperature change during development are often similar to those produced by mutant genes when the temperature is constant. In other words, an individual of one genetic constitution raised at one temperature resembles phenotypically an individual of a different genetic constitution raised at another temperature.
3. The time of appearance of phenocopies agrees with facts already known about temperature-effective periods (TEP's) in the ontogeny of *Drosophila*.
4. The temperature-effective periods for different characters may vary in duration and may occur at different periods in development.
5. Some TEP's occur before the morphological ontogenetic processes affected.
6. Other TEP's are specific for the morphological developmental period of the organ concerned.
7. A number of cases of reversal of dominance in varying degrees are presented.
8. The degree of dominance of a gene is a function of the environment within which the developmental processes take place.
9. The production of phenocopies and the reversal of dominance are considered in the light of the current rate theory of development.

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SOMATIC EFFECTS OF TEMPERATURE ON DEVELOPMENT IN *DROSOPHILA MELANOGASTER*. II. TEMPERATURE- EFFECTIVE PERIOD OF TRUNCATE

(One figure)

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PHENOTYPIC reversal of dominance in *Drosophila* as a result of temperature treatment was first reported by Goldschmidt (1935) for the genes ebony and vestigial. Since then, Child, Blanc, and Plough (1940) have demonstrated that the phenotypic expression of many nominally recessive genotypes could be induced in heterozygotes of *Drosophila melanogaster* by means of a 12-hour exposure to 36° C. at fairly definite periods during larval and pupal development. They considered that these sensitive periods represented developmental or physiological stages in growth during which the rates of certain "gene-controlled reactions" could be influenced by adequate variation of the temperature environment. Two of the authors have conducted further experiments in an effort to determine accurately the temperature-effective periods (hereafter called "TEP") for one or more characters. Puparium formation was chosen as a definitive stage in developmental physiology, and the pupae were subjected to heat treatments during the 36-hour period immediately following this point in development. Special study has been made of the phenotypes truncate wing and vortex, both of which are phenotypic expressions of the genotype dumpy (dp).

MATERIALS AND METHODS

The incubators used were the two-shelf Bridges type, maintained at temperatures which were constant at any one point within $\pm 0.2^\circ \text{C}$. They were placed in a cold room in which the temperature was held at $15^\circ \pm 2^\circ \text{C}$. at a relative humidity of 65 ± 5 per cent. This equipment has been fully described in *Drosophila Information Service*, Bulletin 6. A wild stock (Florida +10), inbred for 1 year by sib-matings and selected for uniformity, was used in these experiments. This was crossed with a 2ple stock which had not been inbred. The control temperature for the first few experiments was held at 25°C . The high temperature used for heating was 36.5°C ., to which larvae and pupae were subjected for a period of 12 hours beginning at 5-5½ days after egg-laying.

Egg-laying periods were, for the most part, 2-3 hours in length. A shorter period cut down considerably the number of eggs obtainable. Best results were obtained by allowing females to lay and larvae to develop in bottles. The bottles retained heat longer than did vials when removed from the incubator. Yeast was added from time to time to keep the supply in the bottles plentiful. Child (1935a) has observed that under these conditions no effects of crowding can be observed with as many as three hundred larvae per half-pint bottle, while, on the other hand, a similar environment and a more homogeneous population result from more individuals in fewer cultures. However, bottles should not be used for heating experiments, since vials, as already noted, react more

quickly to changes in temperature. Various methods were used to collect and count eggs. Best results were obtained by keeping mass matings of 4-7-day-old flies in half-pint bottles inverted over milk-bottle caps on which fresh food had been smeared. A thin, flexible scalpel was used to transfer the pupae from the culture bottles to 2 per cent agar slants. Transfers were made every 2-4 hours.

EXPERIMENTAL

The fact that truncate and vortex—pleiotropic effects of the gene dumpy—are practically unique in heterozygous 2ple stock has already been described (Child, Blanc, and Plough, 1940) as a case of reversal of dominance. The TEP for this phenomenon was localized between 4.5 and 5.5 days, or just after the time of puparium formation. The accuracy of this determination, however, was conditioned by two observations. (1) Variability in extent of effect was noticed, suggesting that individual flies were re-

TABLE 1
RELATIONSHIP BETWEEN TEP FOR TRUNCATE AND TIME OF PUPATION: MALES
(Ratio of flies showing truncate effect to total number of flies in group)

TOTAL AGE IN HOURS	NUMBER OF HOURS SPENT AS PUPAE BEFORE HEAT TREATMENT																		TOTAL NUMBER OF FLIES
	6-9	6-12	7-12	8-10	9-12	10-12	11-15	12-14	12-15	12-15½	14-16	15-18	15-19	16-18	16-20	18-20	20-22	19-23	
118-22	0.4	...	0:10	...	12:12	10:12	0.4	...	0:2	22:44
116-26	2 3	12:13	0.15	0:16	...	14 17
120-24	0.4	...	2:17	...	11:11	12:21	0 1	...	0:2	0:1	...	25 61
120-26	1:18	0:18	30:52*	0:14	40:102
122-26	1 1	11:15	0:7	...	0:4	12:34
121-29	0:8	10:19	11:16	...	0:6	...	30:40
119-33	...	4:15	9 12	13 27
Total number of flies	1:18	4:15	2:3	0:8	0:26	2:27	21:25	24:24	30:52*	10:19	33:48	0:14	0:15	0:15	11 16	0:11	0:11	0:16	...

* Pointed wings not included in this group.

ceiving different amounts of treatment. This might mean that these individuals were subjected to heat treatment during different portions of the TEP. (2) The data were tabulated according to total age of flies at time of treatment. This introduced an element of error in timing, for it was noted that egg-larval age of flies developing at 25° C. from eggs laid in a 2-3-hour period varied as much as 24 hours in length.

It may be suggested then that flies of the same chronological age differ in developmental or physiological age. Since the TEP for truncate occurs in early pupal life, it was deemed likely that calculation of the TEP in terms of pupal age would reduce to a minimum the variation introduced by differences of physiological age in a population. To this end, a series was run in which an accurate check was kept of both total age and pupal age at time of treatment.

Tables 1 and 2 show the relationship between the TEP for truncate and time of pupation. The age of the flies at the beginning of the period of heat treatment is represented in hours; total age from time of egg-laying is represented on the vertical axis; and pupal age, or age from time of puparium formation, is indicated on the horizontal axis. Each fraction represents the ratio of flies showing the truncate effect to total number of flies in the group.

The data show that the TEP for truncate is more closely related to pupal age than to total chronological age, i.e., age from time of egg-laying. Truncates are found throughout when the data are tabulated on the basis of total age, whereas the period of effect is largely localized at 12-16 hours of pupal age.

This type of correspondence is to be expected. As Child (1935b) and Margolis (1935b) have observed, a TEP for a character in a population is the sum of two quantities: the TEP's in the individuals, and the variation from fly to fly in the time of occurrence of their TEP's. Thus, flies developing at 25° may reach the period of puparium formation within a time range of $4\frac{1}{2}$ - $5\frac{1}{2}$ days (Tables 1 and 2). This spread in time of development has been noted by many investigators and has been carefully and extensively studied by Powsner (1935). He attempted to reduce this variability by extreme control of temperature, food, and other environmental conditions and by using a stock inbred and

TABLE 2
RELATIONSHIP BETWEEN TEP FOR TRUNCATE AND TIME OF PUPATION: FEMALES
(Ratio of flies showing truncate effect to total number of flies in group)

TOTAL AGE IN HOURS	NUMBER OF HOURS SPENT AS PUPAE BEFORE HEAT TREATMENT																	TOTAL NUMBER OF FLIES
	4-8	6-12	7-11	8-10	9-12	10-12	11-15	12-14	12-15	12-15½	14-16	15-18	15-19	16-18	16-20	18-20	20-22	
118-22.	0:10	...	1:11	...	14:16	0:11	24:48
116-26	0:7	2:6	10:19	0:17	0:11	12:00
120-24	1:6	...	2:14	...	15:16	2 7	0:6	0:1	20 50
120-26	1:44	10:40*	0 13	10 97
122-26	0:1	...	1:3	1:11	0:6	...	0 7	0:3	2 31
121-29	27:30	1 11	28:41
119-13	...	2:20	1:9	10 12	13:41
Total number of flies	0:7	1:26	1:9	1:16	1:4	3:26	20:31	30:35	18:40*	27:30	12:20	0:13	0:17	0:12	1:11	0:7	0:4	0:11

* Pointed wings not included in this group.

selected for 3 years. The residual variability was still present. Under these conditions, flies of the same chronological age may differ in physiological age; and, conversely, flies of the same pupal age may differ as much as 24 hours in actual age. Pupation is a physiological landmark in the development of the organism. A TEP is a specific time in the developmental physiology of the organism during which certain thermolabile developmental processes occur. A much closer relation holds, therefore, between TEP and pupal age than between TEP and total chronological age.

TRUNCATE

The data may therefore be tabulated according to pupal age at time of treatment. This has been done in Table 3. The truncate effect is variable; different grades of wing shape may be found, varying from nearly normal through a simulation of the phenotype of the oblique allele to a dumpy as extreme as the wings of homozygous dumpy flies raised at 25° C. In this table the truncates have been separated into three groups, representing three degrees of effect—pointed, truncate and pointed, and truncate (Fig. 1).

Pointed wing, with the same TEP as truncate, seems to be an intermediate step between truncate and normal wing. The inner distal third of the wing is flattened

obliquely. It is a variable character, merging into normal and showing gradations toward slight truncate. That it is a partial truncate is further borne out in that it appears where a less marked truncate is expected—e.g., in females, in heat-treated stocks not carrying dumpy, and in stocks heated less than 12 hours.

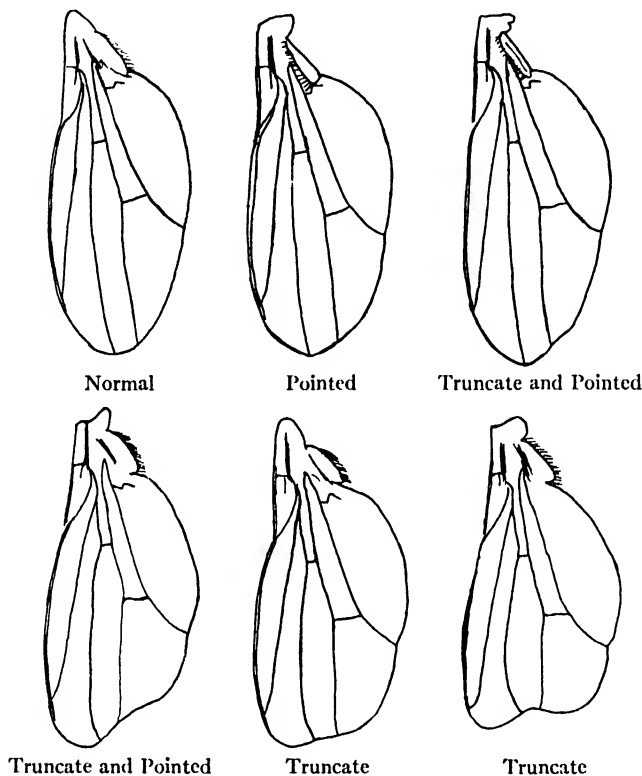


FIG. 1.—Normal and certain mutant wings in *Drosophila*

TRUNCATE AND POINTED

The third category in Table 3 includes those flies in which both the truncate and pointed patterns could be discerned.

The results included in Table 3 show that the TEP for truncate begins at 12–16 hours of pupal life. Some truncates have been obtained in earlier periods, while the sensitive period for one group of flies occurs a few hours later. But the body of evidence substantiates the 12–16-hour TEP.

Whether or not the TEP is identical for males and females cannot be ascertained from the data. A difference in degree of effect, however, is apparent. The truncate effect is qualitatively and quantitatively greater in males than in females. The percentage of occurrence of truncate and pointed is 100 per cent for males and 86 per cent for females in the 12–14-hour period, and 68.8 per cent for males and 41.4 per cent for females in the 14–16-hour period. Considering the two phenotypes separately, in the 12–14-hour period 100 per cent of the males show truncate and 8.3 per cent of them show

pointed, while 68.6 per cent of the females show truncate and 28.6 per cent show pointed. In the 14-16-hour period 64.6 per cent of the males show truncate and 27.1 per cent show pointed, while 27.6 per cent of the females show truncate and 20.7 per cent show

TABLE 3

DEGREE OF MANIFESTATION OF TRUNCATE

(Relation between age as pupae at time of treatment and number of pupae affected)

PUPAL AGE BEFORE HEAT TREATMENT (IN HOURS)	FEMALES				MALES			
	Truncate	Truncate and Pointed	Pointed	Number of Pupae Treated	Truncate	Truncate and Pointed	Pointed	Number of Pupae Treated
6-10.....			I	7				3
7-11.....	I		I	13	5			13
8-10.....	I			16				9
9-12.....				8				12
10-12.....	3			26	2			27
11-15.....	11		8	30	18	I	2	25
12-14.....	20	4	6	35	22	2		24
12-16.....	22	4	2	31	17	1	1	19
14-16.....	6	2	4	29	20	11	2	48
15-19.....				21			I	19
16-18.....				12				15
16-20.....		I		11	10		1	16
18-20.....				7				11
19-23.....				12				16
20-22.....				5				11

TABLE 4

GRADES OF TRUNCATE FOLLOWING TREATMENT AT DIFFERENT AGES

(In percentage)

GRADES OF TRUNCATE IN DESCENDING ORDER OF MAGNITUDE	PUPAL AGE AT START OF TREATMENT			
	12-14 Hours		14-16 Hours	
	Females	Males	Females	Males
Truncate (maximum).....	66.7	91.7	50.0	60.6
Truncate and pointed (intermediate).....	13.3	8.3	16.7	33.3
Pointed (minimum).....	20.0	0.0	33.3	6.1
Total percentage.....	100.0	100.0	100.0	100.0

pointed. Equating at 100 per cent the total number of flies showing an effect, the relationships are as shown in Table 4. With pointed considered as a minimal grade of truncate and with flies showing both pointed and slight truncate as intermediates, it is clear that the truncate effect is more pronounced, i.e., qualitatively greater, in males than in females.

VORTEX

Vortex, another phenotypic expression of dp, also appeared as a result of heat treatment of heterozygous 2ple stock. Its TEP is earlier than that of truncate, i.e., beginning at approximately 8–10 hours of pupal life. The data are summarized in Table 5. The phenotype varied from slight whorling of microchaetes to distinct humps on the thorax. Vortex also appeared in the treated heterozygous crossveinless (cvc in chromosome 3).

The appearance of vortex in a group of treated flies was associated with the occurrence of a number of undeveloped or abnormal pupae. These were similar to the series described by Plough and Child (1936). They included flies with only the thorax, with head in thorax, etc., as well as a number of "white" pupae, in which nothing but a white viscous liquid was found surrounding a number of unhistolyzed larvae organs.

Vortex also appeared in a series in which the pupae were kept at 18°C. from the time of pupation to the time of heating. (Otherwise, 25°C. was used as the control

TABLE 5
TEP OF VORTEX
(Relation between age as pupae at time of treatment
and number of pupae affected)

PUPAL AGE IN HOURS	FEMALES		MALES	
	Vortex	Number of Pupae Treated	Vortex	Number of Pupae Treated
0-4.....	0	2	0	4
4-8.....	3	3	0	2
6-9.....	1	6	1	10
6-10.....	0	4	0	1
7-11.....	0	12	0	11
8-10.....	4	16	3	9
8-12.....	2	51	2	20
9-12.....	1	8	0	12
10-12.....	0	26	0	27

temperature.) On the basis of Powsner's (1935) figures for length of pupal period at these two temperatures, 1 hour at 25°C. equals approximately 2 hours at 18°C. It was found that vortex appeared at ages comparable to expectation on the basis of the results obtained at 25°C., viz., at 14–20 hours.

Most of the data discussed were obtained with the use of a 12-hour heating period. A heating period of 15 hours gave similar results, which have been included with the foregoing. Heating periods as brief as 6 hours were tried. Truncate was induced with a treatment of 9 hours, while 6 hours sufficed for manifestation of slightly pointed wing. Treatments of less than 12 hours showed the same TEP at 12–16 hours of pupal life.

Wild-type males, heterozygous 1ple females, and heterozygous cvc males and females were also treated in these experiments. Pointed wing appeared in all these stocks at 12–16 hours as a result of periods of heat as brief as 9 hours. Vortex appeared in heterozygous cvc as a result of a heat period of 12 hours.

Other phenotypes were observed to have TEP's beginning in the first 26 hours of pupal life. They were: partial crossveinless, missing microchaetes, plexus, and certain bristle effects, including forked, singed, bent, and bifurcate. In some cases microchaetes

were almost completely removed. Plexus affected different cells of the wing, depending on the time of heat treatment. All these effects were specific in point of time. Induced as a result of heat treatment of 12 hours in duration, they showed sensitive periods which began within limits of 4 hours. These data will be elaborated elsewhere.

THE TRUNCATE REACTION

Certain indications are forthcoming as to the nature of the process leading to the expression of heterozygous dumpy. Auerbach (1936) has described in general terms the development of the dumpy wing. From the eversion of the wing pouch at approximately 6 hours after puparium formation to about 17-20 hours of pupal age, the wing seems to develop normally. It presents at the latter time an oblique flattening of the inner distal end of the wing, which is similar in appearance to the slight pointed effect of truncate. From this time on, the wing is subject to a progressive truncation, until it assumes its characteristic shape completely at about 35 hours of pupal life. According to Goldschmidt (1937), this effect is the result of degeneration and resorption of the subepithelial tissue toward the margin of the wing.

We may draw two inferences from these embryological observations: (1) the last step in the series of processes leading to truncated wing is a visible degeneration of already formed wing material (Goldschmidt, 1937); (2) there is a close temporal relationship between this degenerative process and the truncate reaction. In fact, the TEP for truncate overlaps the visible ontogenetic process of wing truncation. The possibility that both reactions are identical may not be overlooked, but for the present it remains in the field of speculation.

Further indications concerning the nature of the truncate reaction are furnished by the phenogenetic evidence. On the basis of the data presented in this paper it may be stated that heat treatment must begin 12-16 hours after puparium formation in order to produce the truncate effect. When begun at this time, heating periods of 9, 12, and 15 hours gave positive results. On the other hand, when begun in the 9-12-hour period, 12- and 15-hour treatments produced very few truncates, which probably belonged to the latter part of the period.

The type of explanation advanced by Margolis (1935*a*, 1935*b*), and later simplified (Margolis and Robertson, 1937) to describe the process of reduction of facet number, would serve equally well as a model for the truncate reaction. According to this scheme, two wing reactions (at least) are involved: (1) a wing-forming process and (2) a truncate-producing process. These are present in the normal, as well as the mutant, fly. When the first reaction exceeds the second, normal wing ensues. When the second outdoes the first, truncate results.

But the size and shape of the eventual wing depends not only on the relative rates of these reactions but also on the length of the time interval in which the latter take place. In other words, the process of general development, i.e., development of the organism as a whole, sets time limits. Therefore, the temperature treatment which produces truncate may act by affecting either or both of two processes. It may increase (or decrease) the rate of the truncate-producing reaction, or it may increase (or decrease) the effective time of reaction.

In terms of the rate theory of development the wing reactions presumably have differing temperature coefficients. When the dumpy gene is present, the temperature coefficient of the truncate-producing reaction is higher than that of the building-up process, so that, as a result of temperature treatment, the degeneration-rate of the reac-

tion may surpass the rate of the building-up process. The result would be more wing substance (or precursor thereof) destroyed than formed during the TEP. It should be borne in mind that the nature of the result may be determined by the relative value of the temperature coefficient for development as a whole. Other mechanisms may also apply. If actual degeneration of wing tissue is not involved, another explanation must be sought.

SUMMARY

1. Study has been made of the characters truncate and vortex, phenotypic expressions of the second chromosomal gene dumpy, which were manifested in heterozygous 2ple stock as a result of high temperature treatment subsequent to the time of puparium formation.

2. The temperature-effective period (TEP) for truncate is more closely related to pupal age than to total chronological age.

3. The TEP for truncate begins at 12-16 hours of pupal life.

4. Truncate wings present a continuous variation from normal to extreme dumpy.

5. The truncate effect is quantitatively and qualitatively greater in males than in females.

6. The TEP of vortex begins at approximately 8-10 hours of pupal life.

7. Heating periods of 12 and 15 hours give similar results, while shorter periods give less pronounced results.

8. Stocks other than heterozygous 2ple showed a slight truncate effect as a result of heat treatment during the same 12-16-hour period, while vortex appeared in one of these stocks.

9. Other phenotypes—partial crossveinless, missing microchaetes, plexus, and certain bristle effects—were observed to have TEP's beginning in the first 26 hours of pupal life.

10. The nature of the truncate reaction was discussed on the basis of the known embryological and phenogenetic facts, as interpreted from the point of view of the rate theory of development.

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THE HEAT PRODUCTION AND OXYGEN CONSUMPTION OF PUPAE OF *GALLERIA MELLONELLA* AT DIFFERENT CONSTANT TEMPERATURES

(One figure)

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IN A number of insects the rate of respiratory metabolism is not constant throughout the pupal period but changes in a characteristic manner. The relatively high rate early in the pupal life decreases to a minimum and then rises, giving a U-shaped curve for the respiratory metabolism during this period. This type of curve for oxygen consumption and for carbon dioxide production has been reported by numerous investigators. Taylor (1927), Taylor and Steinbach (1931), and Crescitelli (1935), in connection with such curves, have noted a terminal drop in metabolic rate just before emergence in *Galleria mellonella*. Krogh (1914) and Janda and Kocián (1933) made studies concerning the effect of temperature on the metabolism of *Tenebrio molitor* pupae, and they conclude that in this species the effect of increasing temperatures is to shorten the duration of the pupal period and to raise the general level of the curves. Krogh's results indicate also that the total amount of carbon dioxide produced during the pupal period of *Tenebrio* is the same at all temperatures studied. Crescitelli (1935) investigated the effect of certain temperatures between 20° and 39° 97 C., inclusive, on the respiratory metabolism of *G. mellonella* pupae; he also found that increasing temperatures tend to elevate the level of the metabolism curves and, within certain limits, to shorten the length of the pupal period. However, his results indicate that the total amounts of oxygen consumed and of carbon dioxide produced are not the same at all temperatures but that there is an optimum temperature (30° C.) at which development is accomplished with a minimum total gaseous exchange.

The heat production of insect pupae has been less extensively studied. Bialaszewicz (1933) found that in *Lymantria dispar* the rate of heat production during the pupal period decreased to a minimum during the first half of the period and then increased, giving a U-shaped curve for the rate of heat production throughout pupal life. Balzam (1933) reported rates of heat production and oxygen consumption during the metamorphosis of *L. dispar*. He obtained U-shaped curves from both types of measurements and found an approximate parallelism between the two. Taylor and Crescitelli (1937), working with *G. mellonella*, have obtained rate curves for heat production which approximately parallel those for oxygen consumption during metamorphosis, both types being of a U-shape, followed by a marked drop near the time of emergence.

There seems, however, to be no literature concerning the effects of temperature on heat production during insect metamorphosis. Consequently, it seemed advisable to make such a study in conjunction with a study of the respiratory metabolism of *G. mellonella* during metamorphosis, and to relate it, if possible, to the measurements of heat production and oxygen consumption of this animal at 30° C., reported by Taylor and Crescitelli (1937).¹

¹ This paper was presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy at Brown University. The writer wishes to acknowledge her indebtedness to Professor Ivon R. Taylor for valuable advice and assistance.

MATERIALS AND METHODS

The bee moth, *G. mellonella*, which was used in this investigation, was obtained from the stock which has been cultured at the Brown University laboratory, and the pupation time of each of the animals employed was known to within an hour. The rates of heat production and of oxygen consumption of individual pupae, at a particular constant temperature, were measured alternately throughout the entire period from the time of pupation up to the time of emergence. The studies were made at each of the following temperatures: 25°, 35°, and 40° C., which were obtained by the use of a constant-temperature water bath arranged so as to maintain each temperature to within ± 0.015 C.

The apparatus and method described by Taylor and Crescitelli (1937) were used in making measurements of heat production of individual pupae during development. Sensitive differential manometers, calibrated by the formula method given by Dixon (1934), were employed in making the oxygen-consumption measurements. The routine procedure used in making the latter measurements was essentially similar to that described by Taylor and Steinbach (1931). As soon after pupation as possible (from 1 to 5 hours), each animal was removed from its cocoon, weighed, and the sex noted. Usually two animals were selected at the same time, and the heat-production measurements were made on one simultaneously with the oxygen-consumption measurements of the other; the animals were then alternated. Such measurements, each covering a period of 30 minutes, were made at about 8-hour intervals throughout the pupal life of the insect. Between measurements, as well as during them, the animals were maintained at the constant experimental temperature under consideration. The loss of weight of the animals was determined by weighing every 24 hours.

The procedure outlined above has been followed in the measurements of heat production and oxygen consumption of *G. mellonella* pupae at three constant temperatures: at 25° and at 35° with ten males and ten females at each temperature and at 40° using six animals of each sex.

RESULTS AND DISCUSSION

THE MEASURED RATES OF HEAT PRODUCTION AND OXYGEN CONSUMPTION

The individual and composite curves obtained from these measurements are very similar in form to those obtained at 30° C. by Taylor and Crescitelli (1937). The values of Taylor and Crescitelli's curves for heat production and oxygen consumption fall, for the most part, between those obtained here at 25° and 35°, as is to be expected. In regard to their heat-production measurements, however, Taylor and Crescitelli have pointed out that, although the pupae have chitinous cases, there is probably some loss of water through the spiracles, and that this evaporation would tend to lower the values obtained when the heat production was measured. They further suggest that, from data on the decrease in weight of the animals and the loss due to elimination of carbon dioxide, it would be possible to estimate the quantity of heat used in this evaporation. In the present work such corrections for heat-production measurements have been calculated; and in doing this, several interesting observations have been made. These will be mentioned in connection with the procedures involved in making the corrections.

CORRECTION OF HEAT-PRODUCTION MEASUREMENTS FOR THE LOSS
OF HEAT OWING TO EVAPORATION OF WATER

a) *Rates of weight loss.*—Since the animals were weighed every 24 hours, it was possible to plot individual curves, and consequently composite curves representing the weight of the pupae at various stages of development. Table 1 shows the average percentage of weight lost in each sex at each of the experimental temperatures. These data indicate that the average percentage of loss in the females is slightly greater than in the males at all of the temperatures studied. More significant, perhaps, is the effect of the high temperature (40°) as seen here. An increase from 25° to 35° does not seem to alter the weight loss greatly, but an increase from 35° to 40° results in a considerably greater percentage of weight lost in both sexes.

TABLE 1
AVERAGE PERCENTAGE OF WEIGHT LOST
DURING PUPAL PERIOD

Temperature (°C.)	Males	Females
25.....	16.1	19.8
35.....	15.0	18.9
40.....	23.7	26.5

The composite weight curves were converted into curves showing the rates of weight loss throughout metamorphosis, in order to apply them in the correction of the heat-production rate curves.

b) *Rates of weight gain owing to respiration.*—Aside from the evaporation of water, probably the only other factor involved in change in weight of the pupae is respiration. If the respiratory quotient of the animal were one, the volumes of oxygen consumed and carbon dioxide given off would be the same, and the weight change would be equivalent to the difference in density of the two gases multiplied by the volume of oxygen consumed. Thus, if the R.Q. were one and the volume of oxygen consumed per unit of time were a liter,

$$\text{R.Q.} = \frac{\text{Vol. CO}_2 \text{ evolved per unit of time}}{\text{Vol. O}_2 \text{ consumed per unit of time}} = \frac{1 \text{ liter}}{1 \text{ liter}}$$

On the basis of the density of the two gases at 25° C., this ratio at that temperature becomes 1.81105 gm./1.30915 gm. In this case the change in weight on account of respiration would be equal to -0.5019 gm. and would indicate a loss of 0.5019 gm. for the consumption on 1 liter of oxygen. But the work of Taylor and Steinbach (1931) indicates that throughout the pupal period of *Galleria* at 30° C. the R.Q. remains constant at 0.69, and Crescitelli (1935) has shown this value to be independent of temperature. When the foregoing ratio is modified for the R.Q. of the animal, we have the ratio equal to 1.24962 gm./1.30915 gm. at 25° C., and the difference in weight because of the consumption of 1 liter of oxygen would be +0.05943 gm. Thus the animal actually gains in weight, owing to respiration. By reducing this value for weight gain to milligrams and by dividing by the weight (in milligrams) of a liter of oxygen at 25°, the factor representing the change (gain) in weight of the animal because of the consumption of 1 mg.

of oxygen at 25° was obtained. In a similar manner the factor for each of the other temperatures was calculated. By means of these factors the rates of gain of weight have been calculated for the animals at each of the three temperatures.

c) *Rates of evaporation of water.*—The observed change in weight of the animals represents the loss of weight by water evaporation plus the gain in weight owing to respiration. Hence, the actual rate of loss of water from the animals is the sum of the observed rate of weight loss and the calculated rate of weight gain, owing to the respiratory ex-

TABLE 2
AVERAGE TOTAL WEIGHT OF WATER LOST
PER GRAM OF ANIMAL

Temperature (° C.)	Males (Gm.)	Females (Gm.)
25.....	0.176	0.221
35.....	0.161	0.210
40.....	0.261	0.309

change mentioned above. Curves showing the actual rates of water loss throughout metamorphosis for each sex at each temperature were plotted, and the area under each was measured with a planimeter in order to determine the average total weight of water lost per gram of animal. Table 2 shows the results of these measurements. From these data it appears that an increase in temperature from 25° to 35° only slightly alters the average total weight of water lost. However, an increase from 35° to 40° results in a considerably greater loss. During these experiments none of the forty animals kept at 25° and 35° failed to emerge. On the other hand, out of the eighteen selected for measurements at

TABLE 3
AVERAGE TOTAL AMOUNTS OF HEAT (GRAM CALORIES
PER GRAM) LOST BY EVAPORATION OF WATER

Temperature (° C.)	Males	Females
25.....	102.30	128.90
35.....	92.58	121.08
40.....	150.00	177.17

40°, only twelve emerged, although in most cases the development seemed to progress normally up to the time of emergence. Crescitelli (1935) showed that a temperature of 39.97° C. resulted in the retardation of the developmental processes in this insect, but he has not attempted to explain the nature of the deleterious effects produced by the high temperature. In the light of the data shown in Table 2 it seems that, among other things, the high temperature might result in excessive drying and hardening of the pupal coverings. Thus, even should the animals develop normally, they might not be able to escape from the hardened cases inclosing them.

d) *Rates of heat loss because of the evaporation of water.*—The rates of heat loss because of evaporation were calculated from the rates of water loss throughout meta-

morphosis, and the results were plotted. By measuring the area under these curves the average total amounts of heat lost by the evaporation of water were obtained, and they are presented in Table 3.

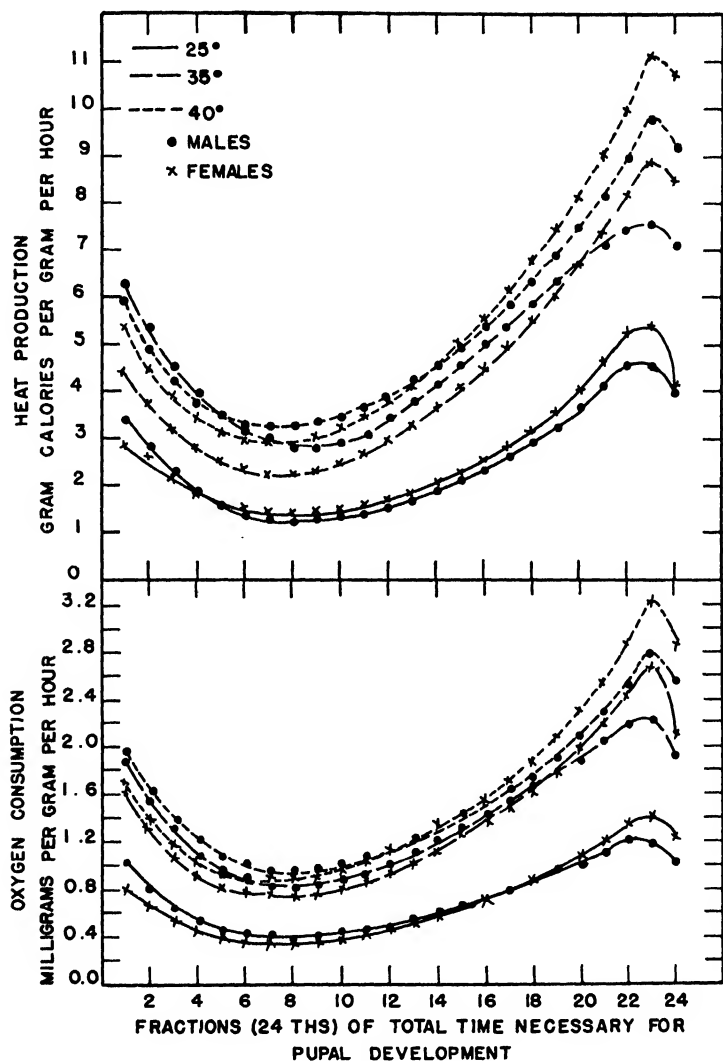


FIG. 1.—Composite curves showing rates of heat production and oxygen consumption for male and female pupae at 25°, 35°, and 40° C.

When the values of the calculated rates of heat loss were added to those of the rates of heat production as measured in these experiments, the values shown in Figure 1 were obtained. In this figure are shown composite curves representing the corrected rates of heat production in gram calories per gram of animal per hour, and the oxygen consump-

tion in milligrams per gram of animal per hour, for each of the sexes at 25°, 35°, and 40° C. Because of the variation in length of pupal life, even in animals of the same sex at the same temperature, it was considered advisable not to plot the composite curves against age in hours but rather to let the total abscissa represent the period of pupal life and the intervals of this abscissa to represent fractions of this period. For convenience, each interval has been arbitrarily chosen to represent one twenty-fourth of the time (in hours) necessary for pupal development. This makes it possible for each composite curve to represent, at all points, comparable stages in the development of the individual animals. In addition, this method of plotting makes possible the direct comparison of composite curves for the different temperatures.

EFFECT OF TEMPERATURE ON HEAT PRODUCTION AND OXYGEN CONSUMPTION

In Figure 1 it is apparent that the typical U-shaped curve with a pronounced drop just before emergence represents the rates of both heat production and oxygen consumption at 25°, 35°, and 40° C. throughout the development of the pupae. Taylor and Crescitelli (1937) found this to be true at 30° C.; and Balzam (1933), working with *L. dispar* pupae at room temperature, reported a U-shaped curve for both heat production and oxygen consumption. The data in Figure 1 further indicate that an increase in temperature from 25° to 35° results in an elevation of the metabolic rates at all stages of development of the pupae. The amount of elevation of each stage, however, does not appear to be constant, being greater during the latter part of metamorphosis than during the early and middle parts. Although it is believed by most workers that both histolytic and histogenic processes occur simultaneously throughout pupal life, the histolytic processes are thought to predominate early in the period and the histogenic processes to predominate in the latter part. Assuming that this is true, there would seem to be some differential effect of this increase in temperature on the two types of processes, as indicated by the heat production and oxygen consumption. The increase in temperature from 35° to 40° apparently has a peculiar effect. During the first part of pupal life at 40° the curves have about the same values as those for the animals at 35°. Soon after the middle of the period, however, an increase in the level of the former becomes apparent, and by the end of development this increase is marked. In the light of the assumption made above, it would appear that this increase in temperature has relatively little effect on the histolytic processes but that it does accelerate the histogenic processes.

EFFECT OF TEMPERATURE ON THE AVERAGE TOTAL HEAT PRODUCTION AND THE AVERAGE TOTAL OXYGEN CONSUMPTION DURING PUPAL DEVELOPMENT

The average total amount of heat produced (including the correction for water evaporation) and the average total amount of oxygen consumed during the entire period have been calculated from the composite curves (Fig. 1) by measuring the area under each. With the data shown in Table 3 the percentage of the corrected total heat production which was used in the evaporation of water was calculated. The results of these calculations are shown in Table 4. In the work of Taylor and Crescitelli (1937) the average total amount of heat produced and the average total amount of oxygen consumed at 30° have not been calculated. However, if it can be assumed that the average total loss of heat due to evaporation of water at 30° is about the same as the average for the three temperatures used here (15.3 per cent for the males and 17.8 per cent for the females), the average total heat production at 30° can be estimated from their curves.

For purposes of comparison this assumption has been made, and the average total heat production at that temperature has been calculated upon this basis and is included in Table 5. Crescitelli (1935) has calculated the average total amounts of oxygen consumed at a number of temperatures, one being 30°05 C., which is practically identical with that (30° C.) used by Taylor and Crescitelli (1937). Table 5 shows a comparison of the data obtained in these three separate investigations.

From these combined data it appears that neither the average total amount of heat produced nor the average total amount of oxygen consumed is constant over tempera-

TABLE 4
PERCENTAGE OF TOTAL CORRECTED HEAT LOST
OWING TO EVAPORATION OF WATER

Temperature (° C.)	Males	Females
25.....	16.3	18.5
35.....	13.9	18.1
40.....	15.6	16.8
Av.....	15.3	17.8

TABLE 5
AVERAGE TOTAL AMOUNTS OF HEAT PRODUCED AND AVERAGE TOTAL
AMOUNTS OF OXYGEN CONSUMED DURING PUPAL DEVELOPMENT

TEMPERATURE (° C.)	HEAT (GRAM CALORIES PER GRAM)		O ₂ (MILLIGRAMS PER GRAM)	
	Males	Females	Males	Females
25.....	625.78	698.04	180.72	191.91
30.....	522.10	629.00	175.84	196.99
35.....	668.44	667.35	195.88	208.67
40.....	960.50	1,057.86	282.10	313.62

tures ranging from 25° to 40° C. Crescitelli concluded that neither the average total oxygen consumption nor the average total carbon dioxide production was constant over a range of temperatures from 20° to 39°97 C., inclusive, but that there was a minimum for each at 30°05. There is good agreement between the results of the present work and that of Crescitelli concerning the average total oxygen consumption at 25°, 35°, and 40°.

THE CALORIFIC QUOTIENT

The calorific quotient is the number expressing the amount of heat evolved in gram calories per milligram of oxygen consumed. Zuntz, Pfluger, and Rubner (quoted by Needham, 1931) have found this number to be 3.2 when protein is burned, 3.3 for combustion of fat, and 3.5 for carbohydrate. The value of this ratio, like that of the respiratory quotient, is supposed to vary according to the particular type of foodstuff undergoing

metabolism. The differences between these numbers, however, are very small; and therefore it is difficult to attach a great deal of significance to calorific quotients obtained in animals experimentally. Nevertheless, in connection with the present work the calorific quotients at various stages of development have been calculated and are presented in Table 6. It will be noted that there is some variation in these values during metamorphosis. Balzam (1933) reported that during metamorphosis of *L. dispar* the calorific quotient values give a V-shaped curve. They diminish at first to a minimum

TABLE 6
CALORIFIC QUOTIENT FOR VARIOUS STAGES OF DEVELOPMENT AT 25°, 35°, AND 40° C.

INTERVALS (FRACTIONS OF DEVELOPMENTAL TIME, IN TWENTY-FOURTHS)	25°		35°		40°	
	Males	Females	Males	Females	Males	Females
1.....	3.31	3.52	3.19	2.66	3.01	3.07
2.....	3.49	3.97	3.24	2.73	3.04	3.18
3.....	3.52	3.88	3.34	2.88	3.06	3.30
4.....	3.46	3.96	3.45	3.02	3.11	3.22
5.....	3.40	3.91	3.58	2.99	3.17	3.26
6.....	3.39	3.87	3.57	2.98	3.23	3.22
7.....	3.28	3.82	3.58	2.89	3.34	3.17
8.....	3.34	3.77	3.42	2.87	3.42	3.17
9.....	3.21	3.78	3.35	2.91	3.43	3.90
10.....	3.25	3.78	3.38	2.93	3.45	3.27
11.....	3.19	3.60	3.39	3.00	3.44	3.25
12.....	3.22	3.53	3.42	3.05	3.46	3.32
13.....	3.10	3.38	3.41	3.13	3.51	3.36
14.....	3.14	3.20	3.47	3.21	3.47	3.40
15.....	3.14	3.37	3.47	3.17	3.50	3.52
16.....	3.16	3.49	3.45	3.23	3.53	3.54
17.....	3.25	3.54	3.51	3.29	3.56	3.57
18.....	3.29	3.68	3.52	3.33	3.59	3.54
19.....	3.44	3.79	3.51	3.35	3.61	3.55
20.....	3.57	3.79	3.52	3.40	3.58	3.50
21.....	3.65	3.90	3.45	3.37	3.54	3.52
22.....	3.73	3.83	3.39	3.36	3.55	3.45
23.....	3.84	3.84	3.37	3.32	3.51	3.43
24.....	3.86	3.25	3.65	3.98	3.60	3.70
Av.....	3.38	3.69	3.43	3.13	3.40	3.39

(2.3) and then later mount to the initial level (3.2) of metamorphosis, the minimum falling toward the middle of the period. However, it appears to the writer that the variation obtained in the present work is neither great enough nor consistent enough to warrant such a conclusion in *G. mellonella* at any of the temperatures employed.

In Table 6 the values for the calorific quotient are considerably higher than those obtained by Taylor and Crescitelli (1937) for *Galleria pupae* at 30°. However, as has been noted earlier, in the work of those investigators the heat loss due to the evaporation of water has not been calculated, as was done in this work; and it seems probable that the values obtained there would be higher if this were done. In connection with the low values which he obtained in *L. dispar*, Balzam suggests that endothermic reactions oc-

curing during metamorphosis might be responsible. However, he makes no mention of the possibility of heat loss due to evaporation of water, and this might also be a factor causing the low calorific quotients which he obtained.

SUMMARY AND CONCLUSIONS

1. Measurements of the heat production and of the oxygen consumption have been made at 25°, 35°, and 40° C. on individual *G. mellonella* pupae throughout development.

2. The measured rates of heat production have been corrected for the heat used in the evaporation of water from the animals. In making this correction the following observations have been made. (a) An increase in temperature from 25° to 35° does not appreciably change the average percentage of weight lost in the pupae, but an increase from 35° to 40° results in a considerably greater loss in both sexes. (b) The change in weight due to respiration is an actual gain, and the rates of such gains at the three temperatures under investigation have been calculated. (c) The rates of loss of water from the pupae are not greatly altered by an increase in temperature from 25° to 35°, but an increase from 35° to 40° results in a much greater loss of water.

3. An increase in temperature from 25° to 35° results in an acceleration of the metabolic processes at all stages of development, but this effect is greatest during the latter part of the period. An increase in temperature from 35° to 40° does not appreciably alter the rate of metabolism during the early part of metamorphosis, but during the latter part of the period the rate is greatly increased.

4. The average total amount of heat produced by the animals at 35° is approximately the same as that produced by animals at 25°; the same was found to be true in the case of the oxygen consumption. In animals kept at 40°, however, there is a much greater average total heat production and oxygen consumption than at either of the other two temperatures.

5. The calorific quotients for various stages of development have been obtained. The average calorific quotients for the pupal period vary from 3.13 to 3.69.

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TEMPERATURE CHARACTERISTICS OF THE ELECTRICAL POTENTIAL IN FROG SKIN

(Three figures)

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A PREVIOUS paper (Barnes, 1939) described the effect of large and abrupt changes of temperature on the electrical potential of frog skin. The present report deals with the effect of more gradual changes in temperature in an attempt to determine the temperature characteristic. The earlier literature is reviewed in the previous paper. It will be recalled that Lesser (1907) believed that the response of the skin to temperature indicated the physical nature of the electrical potential, while Lund and Moorman (1931) obtained a Q_{10} of 2 (based on average potential over 3-hour intervals at 16°4 and 26°4 C.), which supported their theory of a metabolic origin of the potential. Motokawa (1938b) studied the effect of temperature on the action current in frog skin.

METHODS

The skin-holder described in a previous paper (Barnes, 1939) was not used in this investigation, owing to the small volume and the possibility of unequal cooling of the salt solution and the glass core on the inside of the skin.

The skin-holder used in the experiments reported in this paper afforded exactly symmetrical conditions of pressure, volume of Ringer's solution, and oxygen on each side of the skin. It was previously found (Barnes, 1939) that tying the skin on a glass tube with the morphological outside against the glass raises the potential. In the new holder the skin was held between two disks of filter paper, 2.5 cm. in diameter, each having in the center a circular opening 1 cm. in diameter. It was found by Mr. R. J. Coe (who will publish at a later date on mechanical factors affecting the potential) that the potential was stimulated if the ring of filter paper was omitted on the side pressing on the morphological outside surface of the skin. The ends of two cylindrical glass chambers each containing 25 cc. of oxygenated Ringer's pressed against each side of the skin held in the filter paper. A round window, 1 cm. in diameter, on the end of each cylinder allowed contact of the Ringer's solution with the skin. A greased glass sleeve held the chambers in place. Silver-silver chloride electrodes (having no electrode potential) dipped into the salt solution on each side. The silver wires were introduced through side arms to prevent contact with the oxygen bubbles, which escaped by separate tubes having glass bulbs to prevent the meniscus from moving up the tubes. The holder was clamped in a water bath, and potential readings were made with a Type K potentiometer. These determinations agreed with curves obtained by switching onto a Micromax recording potentiometer.

The temperature of the bath was lowered by adding ice; but, because of the small magnitude of the average potential change with temperature, increments of 5° C. were used. The rate of temperature change was about 1° C. per minute, and a constant

temperature was maintained for about half an hour to allow the skin potential to come to equilibrium. Dorsal or ventral skins were kept in Ringer's solution of pH 7.5 for varying intervals before the experiment. The weight and sex of each frog and other particulars are recorded in Table 1.

TABLE 1
TEMPERATURE CHARACTERISTICS OF SKIN POTENTIAL

Date	Pre-vious Temperature (° C.)	Sex	Weight (Gm.)	Skin	Age of Preparation (Min.)	First Temperature (° C.)	Length of Time at First Temperature (Min.)	Temperature Range of Run (° C.)	Duration of Run (Hr.)	μ -Value (Cal.)	Potentials during Run (Mv.)	Remarks
Dec. 28	14	Female	48	Dorsal	75	28	40	25-0	4	5,750	31 -12 5	
Dec. 30	13	Female	29	Ventral	35	20-5	5.2	5,060	36 -17	Rose 15-23 mv. at 5°-0°. See Fig. 3
Jan. 13	15	Female	Ventral	5	27	180	20-0	3	5,360	31 -12	Fell 78-34 mv. at 27°.
Jan. 17	13 5	Female	35 5	Dorsal	105	20	60	15-5	2 6	17,250	15 - 5	Slight fall at 20°-15°
Jan. 26	15	Male	26 5	Ventral	20	26-0	5	11,500	22 0- 2 1	Fell 46-22 6 mv. at 26°.
Feb. 17	15	Female	40	Dorsal	180	27 and 20	130	15-5	1 5	5,018	37 2-27 3	Rose 37-40 mv. at 27°-20°
Feb. 18	14	Female	40	Dorsal	75	20	40	15-0	3	{ 5,000 10,323 }	40 -17 7	Low μ above 15°
Feb. 28	15 5	Female	37	Dorsal	25	20	75	15 5	2 5	9,571	31 5-17	Slight fall at 5°-0°.
Mar. 3	16.5	Male	...	Dorsal	125	20	75	15-5	2.2	10,750	26 7 13	Slight fall at 5°-0°.
Mar. 7	17	Female	59	Dorsal	35	20-5	4	9,966	24 -- 8	2 hr. at 20°; slight drop at 5°-0°.
Mar. 9	16	Female	40	Dorsal	80	20-10	4.3	9,775	23 -12 3	See Fig. 1 Slight drop at 10° 5°
Mar. 14	15	Male	38 5	Dorsal	25	20-0	2.7	5,661	19 - 9	Rebound at each temperature. See Fig. 1
Apr. 13	15	Female	42 5	Ventral	45	20	60	15-0	4	9,000	48 -21	Rose 34-48 mv. with eosin at 20°

RESULTS

In spite of the notorious variations in potential, satisfactory curves were secured after the preliminary period of potential fall. Of a total of eighteen graphs obtained, only six had to be discarded, owing to fluctuations, gradual changes in potential occurring independently of temperature, or failure of the skin to respond to temperature changes. It was found that unsatisfactory results occurred at temperatures above 20° C. Engelmann (1872) reported a fall in potential at 25° C. and above. Klopp (1924) obtained sharp falls at 38° C., but he did not study the interval 21°-38° C. The frogs for my experiments were kept in a cement tank containing damp leaves at about 15° C. and were probably "acclimatized" to this temperature. A rise in potential frequently occurred between 28° or 20° and 15° C. Also, when 0° C. was reached, the potential sometimes rose slightly or failed to fall in the interval 5°-0° C. The initial interval of inconsistent potentials at 20° or above is indicated as "first temperature" in Table 1. In most of the preparations the potential approached a constant level, as indicated in Figure 1, A. It was previously found (Barnes, 1939) that a fall in potential produced by rapid cooling of the skin is followed by a "rebound" potential in which about 40 per cent of the lost potential is regained within 15 minutes. These rebound potentials also occurred with gradual cooling in some skins, as shown in Figure 1, B.

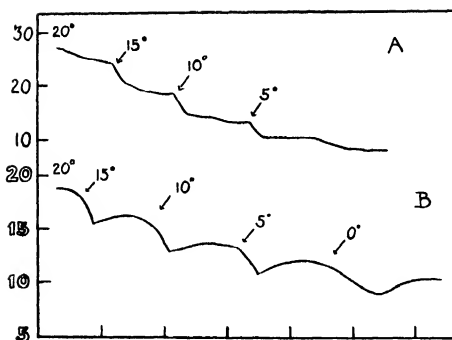


FIG. 1.—Effect of temperature on the electrical potential of frog skin. Ordinates: electromotive force in millivolts (outside surface is negative); abscissas: time in $\frac{1}{2}$ -hr. intervals. *A*, dorsal skin from 59-gm. female, March 7 (see Table 1 and Fig. 2). The temperature changes are indicated at arrows. $\mu = 9,966$ calories (calculated from last potential on "plateau"). *B*, dorsal skin from 38.5-gm. male, March 14 (see Table 1). $\mu = 5,661$ calories calculated from low potentials before "rebound."

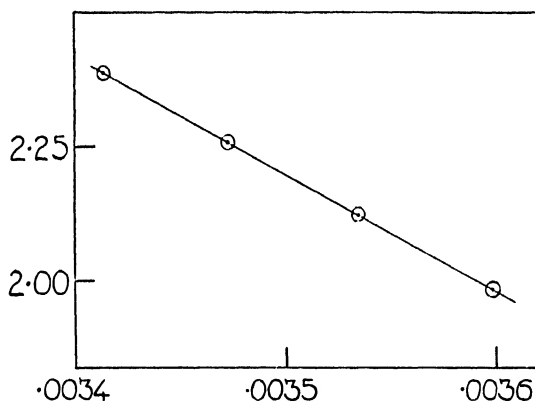


FIG. 2.—Rectilinear relation between logs of potentials (ordinates) and reciprocals of the absolute temperature (abscissas). Dorsal skin of 59-gm. female, March 7 (see Fig. 1, *A*, and Table 1). $\mu = 9,966$ calories.

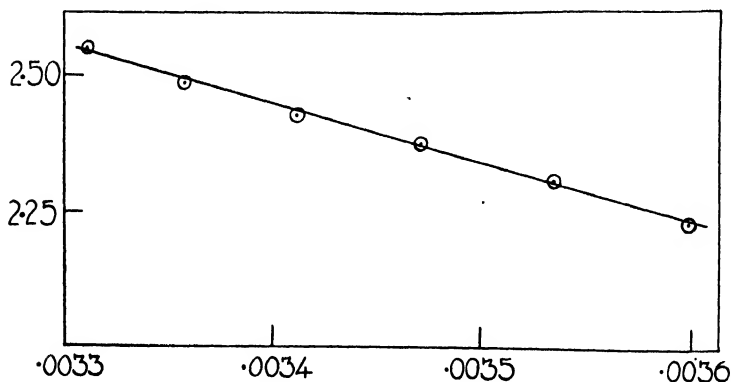


FIG. 3.—Rectilinear relation between logs of potentials and reciprocals of absolute temperature in ventral skin of 29-gm. female, December 30 (see Table 1). $\mu = 5,060$ calories.

TEMPERATURE CHARACTERISTICS

It was surprising to find that an exceptionally rectilinear relation exists between the logarithm of the potentials and the reciprocal of the absolute temperature, as required by the Arrhenius equation:

$$\ln \frac{R_1}{R_2} = \frac{\mu}{2} \frac{1}{T_1} - \frac{1}{T_2}.$$

It was assumed that each potential represents the *rate* of reaction of a process generating the electromotive force. Francis (1933) has demonstrated a continuous current of $20\text{--}30 \times 10^{-6}$ amperes maintained for many hours by frog skin. Of the total of thirteen satisfactory graphs, five yielded a μ -value in the vicinity of 5,000 calories; six yielded a μ of about 10,000 calories (see Figs. 2 and 3). One skin gave a μ of 5,000 above 15°C . and 10,000 below this critical temperature. The μ -value of one skin was much higher than all the others, i.e., 17,250 calories. Motokawa (1938*b*) reported a μ -value of 15,900 for the action current in frog skin stimulated through the cutaneous nerves.

DISCUSSION

The low μ -value of 5,000 calories is not common for other physiological processes (cf. Barnes, 1937); and Crozier (personal communication) suggests that it may be the result of parallel processes, one of which has a high μ but contributes only slightly, while the other may be a simple physical matter, such as ionic mobility. These skins with the low μ -value approach the ideal case of a reversible galvanic cell, as postulated by Lesser (1907), but the potential follows the absolute temperature logarithmically. The low temperature characteristic lends support to the theory that the electromotive force is closely associated with ionic diffusion (cf. Amberson, 1936; Motokawa, 1938*a*; Dean, 1939).

The μ -value of 10,000 calories in certain skins clearly indicates a chemical process as the master-reaction in these preparations. The value is close to the mode of 11,000, which has been associated with hydrolysis. This value occurs in many respiratory phenomena and perhaps represents hydrolysis preparatory to oxidation. The value of 17,000 is close to the mode of 16,800, which is identified with oxidation. Indeed, Krogh's data on the oxygen consumption of the frog yield a μ -value of 17,000 calories (cf. Crozier, 1924). The dependence of the potential on metabolism is shown by the inhibiting action of the following depressants: cyanide, ether, and chloroform (Engelmann, 1872; Alcock, 1907; Lund, 1926); urethane (Boell and Taylor, 1933; Duce, 1936; Ponder and Macleod, 1937); sulphide, carbon monoxide, amyl and butyl alcohol (Francis, 1934); bromacetic acid (Huf, 1936); iodoacetate (Francis and Gatty, 1938); and heavy water (Barnes, 1936, 1939). It has been known for some time that oxygen deficiency lowers the skin potential (Engelmann, 1872; Lund, 1926; Williams and Sheard, 1932; Francis, 1934; Taylor, 1935-36). Also, the striking increase in potential produced by eosin (Barnes and Golubock, 1938) is usually accompanied by an increase in oxidation.

It is possible that the oxidation of large organic molecules releases bound ions, producing a diffusion potential associated with the μ -value of 5,000. If the supply of ions is low, the master-reaction will be the precursor chemical factors indicated by the μ -values of 10,000 and 16,000. The response of the skin potential to temperature may

be modified by the presence of Ringer's solution on the outside of the skin (normally in contact with water). Several workers have localized the potential on the outside (Alcock, 1907; Duce, 1936). The linkage between metabolism and potential is probably very complex. Francis (1933) has calculated that the electrical-energy output is from 5 to 10 per cent of the energy released by oxidation (assuming glucose is burned). Dean (1939) has suggested that the negative charge may be the bicarbonate ion produced in respiration. Dean and Gatty (1937) believe that the diffusion potential is complicated by the mosaic nature of the skin, local-action currents, and ionic adsorption.

The specific temperature characteristic undoubtedly depends on the physiological state of the skin preparation but does not seem to be correlated with weight, sex, or dorsal or ventral surface of the frog (see Table 1).

SUMMARY

1. The effects of slow changes in temperature on the potential of isolated frog skin in Ringer's solution indicate temperature characteristics of 5,000, 10,000, and 17,000 calories.

2. It is suggested that the low μ -value of 5,000 is associated with ionic diffusion, and the higher values with metabolic processes supplying ions as precursors of the potential.

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THE ACTION OF CALCIUM ON MUSCLE PROTOPLASM¹

(One plate)

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TO A cellular physiologist, impressed with the essential similarity of protoplasm in all types of living material, it is rather surprising that the students of muscular contraction have made so little attempt to explain the behavior of muscle fibers in terms of their protoplasm. The muscle physiologist, adept as he is at chemical analysis and at various intricate types of physical recording, has hardly ever considered the fact that the protoplasm of the muscle cell is not markedly different from the protoplasm of less differentiated cells whose physical properties are easier to study. At the present time, although we know that various organic compounds react during or after muscular contraction, no one has been able to correlate any single chemical reaction with the visible fact of muscle-shortening. It is true that there has been some recourse to molecular theories, and at various times Astbury has urged that the contraction of muscle is due to a superfolding of myosin molecules (see, for example, Astbury, 1938).

However, on the whole, it seems clear that the direct attack on the problem of what makes a muscle shorten has not yielded any immediate hope of a satisfactory solution. It is the purpose of this paper to interpret the peculiar power of muscle protoplasm to shorten in terms of our information concerning other types of protoplasm.

According to views previously expressed (for summary see Heilbrunn, 1937, chaps. ix, and xxxvii), cells typically consist of a cortex which is a gel, and a more or less fluid interior. Among its other constituents, the cortex contains calcium in a bound form. On stimulation calcium is released from the cortex and enters the cell interior. There it produces gelation or clotting. This clotting reaction is essentially the same type of reaction as that which occurs when naked protoplasm is exposed by tearing or cutting a cell (surface precipitation reaction). (For further details of the theory and for references to experiments which support it see Heilbrunn, 1937.) If the theory is correct, it is possible to divide the effects of stimulation into two stages: the first involves the release of calcium from the cortex, and the second assumes that calcium produces a reaction in the protoplasm similar to the surface precipitation reaction.

In how far is it possible to apply this theory to the muscle cell? The experiments to be described show that the muscle protoplasm is extremely sensitive to calcium ion and that entrance of calcium into the cell causes pronounced shortening (compare also, Chambers and Hale, 1932). This constitutes evidence in support of the second part of the theory. In a later paper evidence will be presented to show that the irritability of the isolated muscle fiber is, to a marked extent, dependent on the presence of calcium in the medium surrounding it. This, together with the earlier work of Ashkenaz (1938*b*), which showed calcium release on ultra-violet stimulation of isolated muscle fibers, will offer support for the first part of the theory.

Single muscle fibers were dissected out from the adductor magnus muscle of the frog

¹ This paper is the first of a series on isolated muscle fibers. The work is receiving the support of the Rockefeller Foundation.

(*Rana pipiens*). The dissections were made for me by Dr. Eleanor W. Ashkenaz, who in the past few years has developed unusual skill in this technique. During dissection the muscles were immersed in buffered Ringer's fluid (for formula see Ashkenaz, 1938a). In order to free the fibers, they must be cut. At each cut end a plug forms, sealing the end of the fiber. Such plug formation has been noted by pathologists (see, for example, Weihl, 1874 [Weihl's paper may be consulted for earlier references]; Thoma, 1906; compare also Speidel, 1938). It is my belief that the plug which appears at the cut surface of the muscle fiber is due to a surface precipitation reaction of the same sort as that which occurs generally when cells are torn or broken (see Heilbrunn, 1937, chap. ix). Certainly the plug formation seems to be favored by calcium ion, just as the surface precipitation reaction is. And yet, whereas the surface precipitation reaction is prevented by the presence of oxalate, in the case of the muscle fiber, large plugs are formed when oxalate is present. This is a point of some interest and will be referred to again later.

When an isolated muscle fiber is placed in an isosmotic solution of calcium chloride (0.09 M), a sudden change occurs. The fiber shortens rapidly until in a minute or two it is only a fraction of its original length. Under the microscope the fiber, as it shortens, presents an unusual picture. Near each plugged end the cross-striations seem to flow into the plug, there to disappear. As this occurs, the plug lengthens, its substance increasing at the expense of the muscle protoplasm. The advancing plug is preceded by a region in which an increase in diameter of the fiber has occurred. There is, accordingly, from each end of the fiber a wave of thickening, followed by a wave of plug formation. The waves from opposite ends meet, and before long the whole fiber is transformed into a mass of brownish-black plug material thicker than the original fiber and wrinkled in contour. As the muscle protoplasm becomes bit by bit converted into plugs, it loses all irritability and dies. The whole process requires about 2 or 3 minutes for completion. Plate I is a series of photographs of an isolated fiber in an isosmotic calcium chloride solution. The individual photographs were taken at 10-second intervals. In order to show the entire length of the fiber, a low magnification had to be employed. As a result, the photographs show little detail. However, they clearly illustrate the shortening of the fiber and its transformation into plug material.

It is a simple matter to show that the progressive shortening of the muscle substance and its transformation into plug material is due to the entrance of calcium into the plugged cut ends of the fiber. If an isolated fiber is suspended over two small glass rods, then it is possible to apply a calcium chloride solution either to the cut ends or to the uninjured surface along the length of the fiber. If the ends are exposed, shortening and transformation of protoplasm into plug material goes on at about the same rate as it does when the entire fiber is immersed. On the other hand, if the calcium solution is applied along the sides of the fiber, with the ends in Ringer's solution, no rapid shortening occurs. Or rather, no shortening occurs unless the fiber has been injured in the course of preparation. If points of injury exist, these behave just as do the ends, and pluglike material grows out from each injured spot.

These observations indicate clearly that, whenever muscle protoplasm is exposed to calcium chloride, there is a rapid and extensive shortening along the length of the muscle fiber. The progressive course of this shortening was determined by a series of measurements made on individual fibers immersed in unbuffered 0.09 molar calcium chloride solution (pH approximately 6.0). At half-minute intervals, the outlines of the fibers

were sketched with the aid of a projection apparatus. This made it a simple matter to measure the lengths of the fibers at each successive interval. In all, fifty series of measurements were made; but in order to conserve space, only half of these measurements are recorded in Table 1. The figures in the table were obtained by measuring outline drawings in millimeters. Inasmuch as the magnification produced by the apparatus was $40\times$, in order to obtain the true length in millimeters it is necessary to divide the figures given

TABLE 1
EFFECT OF ISOSMOTIC CaCl_2 ON ISOLATED MUSCLE FIBERS

No.	ORIGINAL LENGTH IN RINGER'S SOLUTION (MM)	LENGTHS AFTER IMMERSION IN CaCl_2 FOR—										FINAL LENGTH	TIME FOR COMPLETE SHORTENING		$\frac{\text{FINAL LENGTH}}{\text{ORIGINAL LENGTH}} \times 100$
		Minutes											Min.	Sec.	
		1/2	1	1½	2	2½	3	3½	4	4½	5				
1.	132	112	102	94	...	72	48	48	2	40	36	
3.	152	103	72	59	35	35	1	40	23	
5.	142	95	72	53	40	40	1	50	32	
7.	147	110	105	82	70	60	44	44	2	45	30	
9.	175	115	95	73	64	70	48	48	2	50	27	
11.	100	...	86	72	66	44	44	2	44	44	
13.	250	...	255	...	220	...	158	75	75	3	40	30	
15.	188	148	124	105	88	79	60	65	40	...	40	4	...	21	
17.	174	112	77	55	37	37	1	35	21	
19.	160	102	81	52	35	35	1	55	21	
21.	146	98	80	62	37	37	1	58	25	
23.	171	152	125	112	114	96	81	70	55	40	46	4	5	27	
25.	171	121	97	83	63	48	48	2	25	28	
27.	152	116	87	63	30	30	2	5	20	
29.	111	62	35	25	25	1	25	28	
31.	140	...	67	47	28	28	1	40	20	
33.	106	188	177	160	140	...	113	...	45	...	54	5	...	28	
35.	262	...	212	192	180	161	157	142	138	128	120	10	30	21	
37.	184	...	173	...	160	...	148	84	12	...	46
39.	170	...	83	63	52	50	38	38	3	...	22
41.	102	147	127	108	101	87	82	72	55	41	...	41	4	30	21
43.	143	104	77	...	60	57	46	46	3	...	32
45.	155	134	120	111	106	100	97	91	92	92	88	76	9	...	49
47.	180	132	...	75	82	71	60	46	46	4	...	26
49.	200	150	130	100	86	75	60	39	39	4	...	19

in the table by 40. In three of the twenty-five recorded experiments the shortening process was much longer than in more typical experiments. In these three cases the records for successive lengths of the fiber after 5 minutes' exposure are omitted from the table, and only the final length is recorded. For the twenty-five fibers whose measurements are given in the table, the percentage of final length in terms of the original length is 28 per cent; for the entire series of fifty fibers, the percentage was 27 per cent.

If isolated muscle fibers are immersed in hypotonic solutions of calcium chloride, they also shorten rapidly. Thus, in solutions of half the concentration of isosmotic solutions, or even in solutions of one-fourth the concentration of isosmotic solutions, shortening is

very nearly as rapid as in the isosmotic solutions. Fibers were also placed in hypertonic solutions of calcium chloride. It was thought that such solutions would cause a more rapid and a more complete shortening than the isosmotic solutions. However, in strongly hypertonic solutions, although there was an immediate and rapid decrease in length, the shortening soon stopped, and there was little, if any, further effect. Table 2 shows the final shortened lengths of the fibers in terms of percentages of the original length for various strengths of calcium chloride solution. Each value represents an average of from five to ten determinations, except in the case of the isosmotic solution, in which all fifty determinations are averaged. With moderately hypertonic solutions (i.e., solutions of twice or three times the isosmotic strength) there is extensive shortening, but this becomes progressively less as the concentration of calcium chloride is increased. Sometimes, in strongly hypertonic calcium solutions, after a rapid initial decrease in length, the fiber may, after a minute or two, grow slightly longer. In Table 2 the shortest length of the fiber was taken in calculating the percentages.

TABLE 2
EFFECT OF VARIOUS CONCENTRATIONS OF CaCl_2 ON ISOLATED MUSCLE FIBERS

CONCENTRATION OF CaCl_2	NUMBER OF EXPERIMENTS	AVERAGE OF $\frac{\text{FINAL LENGTH}}{\text{ORIGINAL LENGTH}} \times 100$	AVERAGE OF TIME REQUIRED FOR COMPLETE SHORTENING	
			Min.	Sec.
$\frac{1}{4} \times$ isosmotic	6	33	3	10
$\frac{1}{2} \times$ isosmotic	9	28	2	45
Isosmotic (0.09 M)	50	27	3	13
$2 \times$ isosmotic	8	25	2	12
$3 \times$ isosmotic	8	28	1	29
$5 \times$ isosmotic	10	60	1	7
$10 \times$ isosmotic	5	75	2	30

The fact that muscle fibers do not shorten as much in strong solutions of calcium as they do in weaker solutions is of considerable interest. It may be that osmotic factors are concerned—at least to some extent. However, there appears to be an interesting correlation with the fact that in the sea-urchin egg the surface precipitation reaction, although initiated by the calcium ion, is inhibited by strong concentrations of the same ion (Heilbrunn, 1930).

Strontium and barium chloride behave much like calcium chloride in their effect on isolated muscle fibers. In isosmotic solutions of strontium or barium chloride the fibers shrink in much the same way and in about the same time as they do in calcium chloride solutions. In isosmotic solutions of sodium or potassium chloride there is no shortening at all comparable with that produced by calcium chloride.

Magnesium chloride acts in a remarkable fashion. Some years ago, in attempting to explain the anesthetic behavior of the magnesium ion and its antagonism by calcium ion, I suggested that both ions acted in the same way, except for the fact that calcium was far more potent (Heilbrunn, 1934). It was argued that immersion of cells in solutions of magnesium salts would result in a replacement of the calcium ion in the cortex by the far less potent magnesium ion. That magnesium is far less potent than calcium in causing a

surface precipitation reaction was demonstrated for sea-urchin egg protoplasm. The reaction was produced by calcium in great dilution. To give the same effect, a concentration of magnesium ion at least one hundred times greater was necessary. A similar relation exists in these muscle fibers. When fibers are immersed in magnesium chloride solutions of half the isosmotic concentration, instead of shortening rapidly, as they do in solutions of calcium chloride of similar strength, the fibers typically lengthen slightly. However, in solutions of five times the isosmotic concentration there is an extremely rapid shortening. These preliminary experiments with magnesium solutions will probably serve as the basis for further work in this laboratory.

One strange phenomenon remains to be discussed. That is the action of oxalate ion. It has been noted that, whereas calcium causes a rapid shortening of the muscle, sodium and potassium have no such effect. And yet, when fibers are immersed in solutions of sodium or potassium oxalate, they show a progressive shortening much like that observable in solutions of calcium chloride. It is true that the decrease in length is much slower. Thus, in an isosmotic solution of sodium oxalate it may require 15-25 minutes for a fiber to attain maximum shortening. Aside from the slower speed the oxalate effect resembles rather closely the calcium effect. In oxalate solutions the fiber shortens from the ends, the plugs progressively lengthening until finally all the muscle protoplasm has disappeared into them. Apparently oxalate produces and encourages plug formation much as calcium ion does, and this constitutes a strong contrast from the relation of oxalate to the surface precipitation reaction in typical cells. In all other cells which have been studied, the surface precipitation reaction is prevented by solutions of oxalate salts. Here, then, is a contradiction, for in the frog muscle cell, oxalate seems to act like calcium. Apparently it is a widespread contradiction. For in many types of living systems, in spite of the fact that oxalate removes calcium from solution, it may act like excess calcium in stimulating to activity. If we could explain this peculiar stimulating power of oxalate ion, such an explanation would remove one of the major difficulties in the way of rational explanation of a number of vital activities. For often enough, under slightly varying conditions, oxalate may act now as a stimulant and again as a preventer of stimulation.

I believe that a simple explanation of the calcium-like effect of oxalate ion may be possible. When a cell, such as a sea-urchin egg, is broken into an oxalate solution, the protoplasm immediately mixes with the oxalate solution, and no surface precipitation reaction occurs. But when a muscle cell is torn or cut, probably because of the presence of fibrils, the protoplasm does not exude, and no mixture of protoplasm and oxalate is possible. If now we assume that magnesium ion diffuses more rapidly through the muscle cell than does calcium—and this is not an unreasonable assumption—then in the interior of the muscle cell, at some slight distance from the torn end, the protoplasm would be increasingly rich in calcium as compared to magnesium. In other words, the calcium-magnesium ratio would increase. Such an increase in the calcium-magnesium ratio might well produce effects of the same sort as those produced by an increase in calcium concentration (compare Wilbur, 1939). The result would then be that the oxalate across a barrier could produce the same general type of effect as an increase in calcium ion concentration.

An attempt has been made to test this hypothesis by immersing muscle fibers in magnesium chloride solutions before subjecting them to the oxalate solutions. If the hypothesis is correct, previous exposure to magnesium should delay the action of oxalate.

PLATE I



PLATE I

Shortening of an isolated muscle fiber in isosmotic (0.09 M) calcium chloride. The succession of stages is from left to right, as on a printed page. The first photograph was taken in Ringer's solution; the second, 10 seconds after the addition of calcium chloride. Subsequent pictures were taken at 10-second intervals.

physiological span of 8.7 atm. for hypotonic solutions, as against the span of 3 atm. for hypertonic solutions.

A comparison of the limits of osmotic pressure beyond which cleavage will not occur is interesting. Eggs do not divide in a solution whose osmotic pressure is less than 13.2 atm., which is 8.7 atm. less than that of sea water. In the other direction, eggs do not divide in a solution whose osmotic pressure is greater than 28.7 atm., an increase of 6.8 atm. The latter figure is considerably less than that reported necessary by Vlès and Dragoiu (1921a, 1921b) for the prevention of cleavage of the eggs of a European sea urchin. They state that the osmotic pressure can be raised by 11 atm. (calculated) before cleavage is impaired. However, considerable variation is indicated by the figures of Białaszewicz (1921). He gives the limits for the cleavage of *Echinus microtuberculatus* eggs as $\Delta = \pm 0.63$ and for *Strongylocentrotus lividus* as $\Delta = \pm 0.57$. These values may be compared with $\Delta = +0.73$ and $\Delta = -0.55$ reported here for the *Arbacia* egg.

b) *Salt effects*.—In the case of cation effects, careful distinction must be drawn between physiological and toxic actions. The latter, of course, have no meaning for this investigation. Thus, the sodium ion causes cytolysis, and its effect on the elongation cannot be studied.

Of the three remaining cations, potassium and magnesium permit incipient elongation; calcium prevents it. Whereas incipient elongation is favored by pure solutions of potassium and magnesium, nevertheless the furrowing process may be abnormal in these solutions. Ameboid motion, such as has been described by F. R. Lillie (1902) for the *Chaetopterus* egg, often sets in. Because the shape of the cell becomes ameboid, it is not possible to make quantitative measurements of the magnitude of the incipient elongation in these solutions. As the cell elongates, its shape becomes irregular, blebs form upon its surface, and indentations marking regions of furrow formation appear and disappear asymmetrically at various points on the cell surface. Internally, the ordered movements of the red-pigment granules toward the cell equator are disrupted, and clumping of these granules takes place in the interior of the cell as well as in the cortex. Many of these granules explode and release their pigment to the exterior. The behavior of the mitotic spindle in relation to the churning movements of the cytoplasm is especially interesting. It seems to bend and twist in every conceivable way. Its long axis may often be perpendicular to that of the cell, and it is not uncommon to find the mitotic figure restricted entirely to one of the two daughter blastomeres. The astral rays of the newly forming asters in the daughter cells are usually markedly bent. These observations fit in with the general conception that the spindle is a rigid body. Less frequently cases are met with in which the ameboid cells elongate to an abnormally great extent and then apparently "set" irreversibly. Nuclear divisions, as well as cytosomal cleavage, are entirely abolished. Such cells usually burst through their fertilization membrane.

Experiments with isosmotic KNO_3 , KBr , and KI gave essentially the same picture, indicating that the effects are associated with the cation rather than with the anion. Similar results were obtained with isotonic KCl solutions made alkaline with KOH to pH 8.4 and made acid with HCl to pH 5.8. It is quite obvious that ions that favor cell extension may in themselves be detrimental to the cleavage process as a whole by effecting pathologically the mechanism of furrow formation.

Eggs immersed in an isotonic CaCl_2 solution remain quite spherical, showing no tendency to elongate. Also, there is no furrow formation; i.e., the entire cleavage cycle is reversibly inhibited. Nuclear division continues for some time.

DISCUSSION

There are many theories of the mechanics of cell division. For the older literature the critical review of Meek (1913) may be consulted. Teichmann (1903), in a widely quoted paper, postulated that cortical changes of a colloid-chemical nature were of prime importance. More recently Chambers (1938) and Marsland (1938, 1939) have especially emphasized the role of sol-gel transformations. As stated in the introduction, it is my purpose to analyze the situation from the same standpoint.

From this viewpoint a consideration of the results on cytosomal elongation obtained by immersing the eggs in isotonic salt solutions is of particular significance. This is because the effects of cations on the properties of colloids are very marked. As far as their action on protoplasmic colloids is concerned, salts have been found to act differently on different parts of the cell (Heilbrunn, 1937). Obviously, the reactions of the cell may be regarded as the physiological integration of various interrelated colloidal changes, and the contribution of any one factor to the end-result is extremely difficult to evaluate. Neglecting, for the moment, these general considerations, it is instructive to compare the effects of cations on the viscosity of the cell cortex with their effects on cell elongation. Data from both physiological and embryological sources are generally agreed that the calcium ion causes cortical gelation. Potassium, on the other hand, liquefies the cortex (Heilbrunn and Daugherty, 1932). Magnesium, although capable of forming nondialyzable proteinates (Duce, 1937*a*, 1937*b*), and so presumably likely to cause gel formation, has been found to liquefy the cell cortex (Heilbrunn and Daugherty, 1932). All that this may mean is that, compared to a calcium proteinate cortex, a magnesium proteinate cortex is relatively less viscous.

Evidently the colloid-chemical effects of these ions on the cell cortex exactly parallel their effects on the incipient elongation. I have shown that potassium and magnesium favor elongation, whereas calcium prevents it. From these results the tentative conclusion may be drawn that any agent that has a liquefying action on the cell cortex will permit elongation. Potassium and magnesium are such substances. Conversely, any agent that has a gelating effect on the cortex may be expected to prevent incipient elongation. Calcium falls in this category.

As a matter of fact, the parallelism between the known colloid-chemical effects of these ions on the cortex and their physiological actions can be shown even more strikingly. It has been found that, whereas pure solutions of potassium and magnesium favor incipient elongation, cleavage may be unsuccessful because the cells tend to undergo ameboid movements. This implies that, whereas agents that have a liquefying action on the cortex may favor incipient elongation, these same agents are not necessarily suitable for successful furrow formation. Evidently the furrowing process is not favored by cortical solation.

As a matter of fact, there is at present considerable evidence that the formation of the cleavage furrow is associated with a process of cortical gelation occurring in the equatorial region of the cell. Among such evidences, one may mention Schechtman's (1937) observations on vital staining during cleavage, Chambers' (1938) microdissection experiments, and Marsland's (1938, 1939) experiments on the effect of hydrostatic pressure upon furrow formation. Thus Marsland (1938, p. 63) writes that "the inhibiting effect of pressure upon the cell division is related to an interference with gelation processes which occur in the cortical protoplasm of the wall of the furrow rather than to other factors."

Experimentally, I find that the fewer the number of washings with isotonic solutions of potassium or magnesium the higher the percentage of successful cleavages. Evidently even traces of calcium in the sea water, carried over by incomplete washing, are sufficient to overcome the liquefying action of potassium and magnesium to the extent that furrow formation is normal. As a result, the number of cells exhibiting ameboid motions is considerably reduced. That small traces of calcium can be effective in counteracting the liquefying effects of potassium and magnesium is shown by the work of Heilbrunn and Daugherty (1932, pp. 265-66), where it is pointed out for the plasmagel of *Ameba* that "even 1 part of the calcium chloride solution to a 1,000 parts of the potassium chloride solution is enough to raise the centrifuge value from 20 to 35."

Clearly, two more or less interrelated processes occur in the cytosome during cell division. One is the process of cell extension, which apparently involves a liquefaction of the cell cortex in all regions of the egg other than the equatorial region. The other is furrow formation, which involves a gelation of the cortical protoplasm passing through the spindle axis. Bearing this general picture in mind, it becomes possible to analyze to some extent the experiments on varying the external osmotic pressure.

It has been shown that the action of hypotonic solutions is to increase the incipient elongation, and that of hypertonic solutions to decrease it. In regard to the action of hypotonic solutions, this statement is perhaps misleading, for these experiments, although intended to apply to only one process—incipient elongation—actually pertain to two processes: furrow formation and incipient elongation. It is evident, therefore, that the effect of hypotonic solutions in increasing the elongation may be due to one of two causes. In the first place, hypotonic solutions may *actively* cause the cell to increase in length above the normal value. On the other hand, the effect of decreasing the external osmotic pressure could very well be to cause a delay in the formation of the furrow. Obviously, anything that inhibits the development of the furrow without effecting the elongation mechanism would give higher values for the incipient elongation. The fact that the time required for the completion of the elongation process is greater in hypotonic solutions than in sea water indicates that the latter explanation is probably the correct one.

I conclude, therefore, that hypotonic solutions only indirectly increase the magnitude of the incipient elongation. The manner in which this takes place appears to be by an inhibitive action upon the onset of the constriction process. The fact mentioned previously, that in extremely hypotonic solutions the cell bulges out instead of constricting at the equator, is in line with the foregoing reasoning. Hypertonic solutions apparently act directly to decrease the extension. It does not seem possible, with our present knowledge, to offer an explanation of the quantitative aspects of these experiments.

SUMMARY

1. The value of the incipient elongation (so-called "karyokinetische Streckung") of the *Arbacia* egg at first cleavage has been determined under normal and experimental—variable osmotic and ionic—conditions. In sea water (osmotic pressure = 21.9 atm.) the cell elongates $3.3\ \mu$ (an increase of 4.6 per cent over its original diameter) before the cleavage furrow appears.

2. When the external osmotic pressure is decreased, the value of the incipient elongation is increased. This relationship is not quite linear. Eggs do not divide in a solution whose osmotic pressure is 11.1 atm.

3. Hypertonic solutions reduce the value of the incipient elongation, and eggs do not divide in a solution whose osmotic pressure is 31.2 atm. This indicates that cell extension is as important a factor in cleavage as furrow formation, since a furrow cannot form unless the cell first elongates.

4. Isotonic potassium and magnesium solutions favor incipient elongation; calcium inhibits it. Sodium is so toxic that its effect on the elongation could not be determined. The presence of calcium appears to be necessary for successful furrow formation, for eggs immersed in pure potassium and magnesium solutions exhibit ameboid motions and cleave imperfectly.

5. The data cited have been used as a basis for the interpretation of the physiology of cell division along colloid-chemical lines, as originally suggested by Teichmann many years ago. There appear to be at least two important factors in the division mechanism: elongation and constriction. Incipient elongation seems to be associated with the liquefaction of the cell cortex; constriction, with a gelation of the cortical protoplasm in the equatorial region. Hypertonic solutions act by arresting the elongation process. Hypotonic solutions probably inhibit the constriction mechanism. To a certain extent, elongation is independent of constriction; but the latter process depends upon, and becomes concomitant with, the former at the close of the incipient period of elongation.

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ENVIRONMENTAL FACTORS AFFECTING CYSTMENT IN *WOODRUFFIA METABOLICA*

(Five figures)

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CERTAIN aspects of cystment in the life-history of *Woodruffia metabolica* have already been described (Johnson and Evans, 1939). In that study it was pointed out that this ciliate regularly forms thin-walled cysts for division, that it forms thin-walled cysts in which no division occurs and in which the organism apparently digests its food, and that it regularly forms thick-walled resting or protective cysts in the absence of food. The purpose of the present investigation was to determine, at least in part, the causes of excystment from the resting state and the effects of various environmental factors on such excystment.

In the beginning of this study and throughout a large part of it, we assumed that all resting or protective cysts were alike to the extent that they required the presence of some organic substance in the excysting medium for excystment to occur. In the study referred to above, it was reported that a solution made by boiling dried *Elodea* leaves in balanced salt solution would consistently produce in the neighborhood of 100 per cent excystment. This is in agreement with the results of Taylor and his students in their work on *Colpoda*, where they have found that some organic substance, such as hay infusion or yeast extract, is necessary for excystment. Weyer (1930) reported that an organic substrate was necessary for excystment in *Gastrostyla*. Recently Haagen-Smit and Thimann (1938), in studies made on *Colpoda cucullus*, have found that the excysting activity of hay infusion is due to the salts of the simple organic acids present.

However, as a result of variations in the methods of preparing cysts, another type of resting or protective cyst was produced. This type of cyst can be activated without the addition of any organic substance. Other workers have reported excystment without the addition of any organic substance. Moore (1924) reported spontaneous excystment in *Spathidium*. Darby (1929) found that he could induce *Stylonychia* to excyst simply by adding fresh water to the cysts. Garnjobst (1937) obtained excystment in *Euplotes* upon the addition of tap or distilled water. Quite recently Taylor and Strickland (1938) have reported that cysts of *Colpoda duodenaria* may excyst spontaneously under certain conditions. The appearance of this second type of cyst in our work has necessitated an extension of our studies in order that a comparison of the two types of cysts might be made. The cysts which require the addition of some organic substance for excystment will hereafter be referred to as "stable" cysts. Those cysts which can be activated without the addition of some organic excysting substance will be called "unstable" cysts.

The two types of cysts differ characteristically in appearance, and it is possible to identify each type without question by low-power microscopic examination. Both types possess the same membranes—an innermost endocyst,¹ which is very thin; a mesocyst,

¹ The use of "endocyst" instead of "intimocyst" for the innermost membrane, and "mesocyst" for the middle membrane instead of "endocyst," seems logical, although the other usage is common in the literature.

which is quite rigid; and an outer, glutinous ectocyst. These membranes are shown in Figures 1 and 2. The endocyst is visible only after emergence from the other two membranes. It swells rapidly, and the organism escapes from it in a few minutes. At least one liquid vacuole is present in unstable cysts; such vacuoles are never found in stable

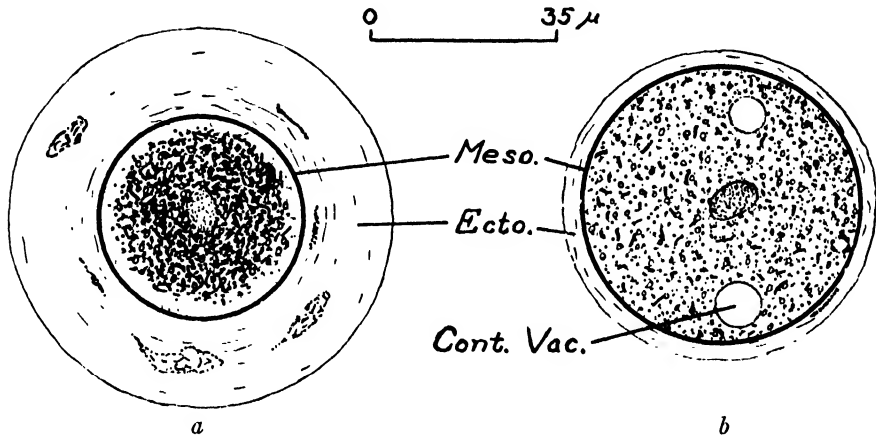


FIG. 1.—(a) Stable and (b) unstable cysts

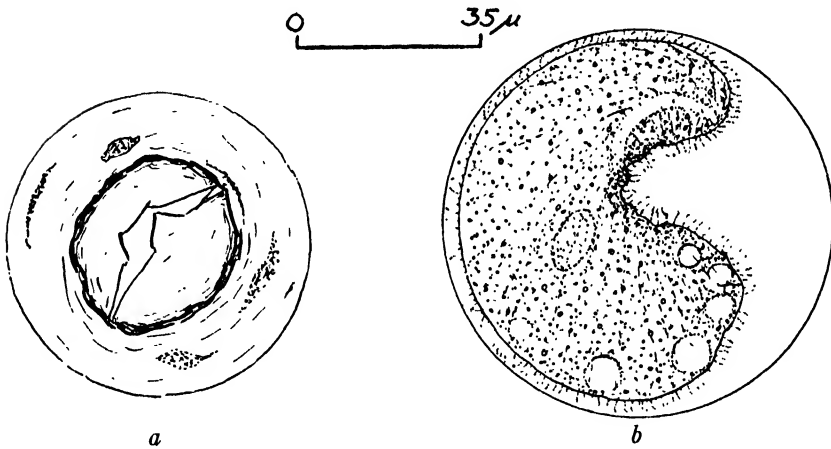


FIG. 2.—Cyst membranes. (a) Empty ectocyst and mesocyst. (b) Organism in endocyst just after emergence from the other two membranes.

cysts. The diameter of the protoplast of unstable cysts is always greater than the diameter of the protoplast of sister-cysts in the stable state. Such a comparison can easily be made in cultures started with a single *Woodruffia* in which both types of cysts develop. Owing to the volume difference, the granules in the cytoplasm of the unstable cysts are less closely packed than are those in the stable cysts. This causes a definite color difference in the two types when viewed under the microscope. These references to strictly morphological differences will serve as an introduction to the two types of cysts.

MATERIALS AND METHODS

The experimental animals used in this investigation were obtained from a clone derived from the original sample collected on the Stanford campus (cf. Johnson and Larson, 1938). *Paramecium multimicronucleata* was used as a source of food. Some of the paramecia were grown in infusions prepared by boiling for 5 minutes $2\frac{1}{2}$ grams of dry timothy hay and 30 grains of wheat in 1 liter of tap water. Such infusion cultures of paramecia were kept in small wide-mouthed bottles. In addition to this source of food organisms, many cultures of paramecia, maintained in balanced salt solution (after Osterhout) with *Pseudomonas fluorescens* as the only source of food, were used. Thousands of paramecia were prepared for use daily by washing at least twice in fresh balanced salt solution by centrifuging. The washed paramecia were then transferred to Columbia watch glasses, and to these a few *Woodruffia* were added. In this way numerous stock cultures of *Woodruffia* were maintained. So long as food is present, division cysts and temporary or digestion cysts are greatly in the majority over free-swimming *Woodruffia*. Upon the depletion of the food, however, all the organisms become free-swimming for a short time. At this cultural phase *Woodruffia* were removed and washed, preparatory to encystment.

Balanced salt solution was prepared with triple-glass-distilled water and C. P. salts (Merck). Johnson (1933) has described the method for preparing the salt solution. Before use, this solution was buffered at a pH of 6.9-7.0. Experimental animals were always washed at least twice in the balanced salt solution.

Columbia watch glasses were used as experimental dishes. Following each experiment the dishes were thoroughly cleaned by scouring, rinsed with distilled water, and dried with a clean towel. Petri dishes were used as moist chambers. For experiments conducted at 15° and 20° C., constant-temperature rooms were used. For experiments conducted at all higher temperatures, incubators were used. Constant-temperature rooms were maintained at $\pm 0.1^\circ$ C., and incubators at $\pm 0.5^\circ$ C.

The method for inducing encystment in *W. metabolica* has been described by Johnson and Evans (1939). However, at that time only the so-called "stable" cyst was recognized, and only after subjecting the encysting organisms to a variety of experimental conditions was the unstable type encountered. The importance of standardization in the preparation of cysts is obvious. A discussion of factors involved in the production of unstable cysts will be given in a subsequent section of this report.

To insure the formation of stable cysts, the cultural history leading to encystment must be considered, though the factors existent at the time of encystment are of primary concern. The following procedure was followed in most cases.

Woodruffia, which had been feeding on large, well-fed paramecia, were washed twice in balanced salt solution by transferring with pipettes. Groups of approximately 30 *Woodruffia* were then removed to culture dishes containing 1 cc. of balanced medium. Unless otherwise stated, the total salt concentration of the balanced medium was 0.012 per cent, which will be designated as "X concentration." These *Woodruffia* were allowed to encyst at 25° C. to form standard cyst preparations.

It is necessary, for experimental purposes, to use cysts which are definitely mature, i.e., cysts which have completed the encystment process. In order to determine at what age cysts are mature, a series of dishes containing free-swimming organisms were prepared for encystment. After the organisms had rounded up and were in the first stage

of encystment, they were placed in an incubator at 30° C. This was considered as zero time; and at regular intervals—namely, 24, 36, 48, 60, 72, and 84 hours—the salt solution was drawn from some of the dishes and the excystment medium added. The average time of excystment in the different dishes was then determined. These results were obtained:

Hours after start of encystment.....	24	36	48	60	72	84
Hours required for excystment.....	27	10	8	7-7½	7-7½	7-7½

Under these conditions the cysts were mature after 60 hours. Similar tests were made with 25° cysts. In these the maturation time was somewhat longer, but in all instances the cysts were mature before the end of the fourth day. In all the excystment experiments, unless otherwise stated, no cysts were used which were less than 4 days old. The excystment time remained the same over a long period of time. The time required for 50 per cent of the organisms to excyst in any given experiment was considered as the excystment time.

In the beginning of these investigations a variety of solutions were tried as to their ability to induce excystment of stable cysts. Plant extracts, such as lettuce, timothy hay, lawn grass, and *Elodea*, would activate the cysts. Quite favorable results were obtained by the use of *Elodea* solutions. During the course of the work solutions of dehydrated alfalfa leaves were tried with excellent results. Solutions of yeast extract, which Taylor and Strickland (1935) reported as giving excellent results with *Colpoda* cysts, were not so satisfactory in this work. The yeast-extract solutions induced excystment, but the growth of bacteria was so much greater than in the other solutions tested that its use was discontinued. Solutions of various vitamins were tried without success. Bacterized gelatin, described by Weyer (1930) as being active on cysts of *Gastrostyla*, was also tried, without results, on cysts of *Woodruffia*.

Both *Elodea* and alfalfa extracts were used as excystment media in the experiments described below. The solutions were prepared by boiling for 3 minutes 3 grams of the dried plant in 500 cc. of buffered balanced salt solution. After filtering, the solutions were autoclaved in test tubes; and, when required, samples were removed with sterile pipettes. In this way the sterility of the solutions could be maintained indefinitely. Any variation in this procedure will be described in respective sections of the paper.

STABLE CYSTS

a) *Concentration of the excystment medium.*—As mentioned above, solutions of dried *Elodea* and of dehydrated alfalfa leaf meal were the best excystment media tried. Table 1 shows the average excystment results obtained in five trials using different concentrations of the two substances on standard cyst preparations.

These solutions were prepared by first making a 0.6 per cent infusion of each substance as described above. Parts of each stock infusion were diluted with balanced salt solution to the concentrations to be used. The test solutions were then autoclaved.

The results given in Table 1 show that the excysting substance must be present in certain concentration to produce maximal excystment and that below a certain concentration, in each case, no excystment occurs. In tests made earlier, solutions of dried *Elodea* in concentrations up to 1.0 per cent had been tried with approximately 100 per cent ex-

cystment in concentrations of 0.6–1.0 per cent. As a result *Elodea* solutions of 0.6 per cent were used in the earlier experiments. The results given here show that the excystment time is a little shorter in the alfalfa solutions and that below 0.6 per cent concentration the alfalfa solutions were more potent than similar concentrations of the *Elodea*. In some of the experiments reported below, a 0.6 per cent alfalfa solution was used; in others, a 0.6 per cent solution of *Elodea*. The kind of excystment medium used in each experiment will be stated. After the alfalfa was first tried, it was used in all later experiments. It is possible to obtain² large amounts of this dried leaf meal, thoroughly mixed and uniform; and in our work over the past six months no change has been detected in its potency.

Thimann and Barker (1934) state that the percentage of excystment in *Colpoda* is dependent upon the concentration of the hay extract used and is independent of the total

TABLE 1
EFFECTS OF DIFFERENT CONCENTRATIONS OF *Elodea* AND ALFALFA AT 30° C.

ELODEA			ALFALFA		
Concentration (Per Cent)	Percentage of Ex- cystment	Excystment Time (Hours)	Concentration (Per Cent)	Percentage of Ex- cystment	Excystment Time (Hours)
0.6.....	98	7.25	0.6.....	100	6.5
0.4.....	80	7.5	0.4.....	98	6.5
0.2.....	74	7.5	0.2.....	95	6.5
0.1.....	55	8	0.1.....	84	6.5
0.06.....	21	8.5*	0.06.....	53	7
0.04.....	20	8.5*	0.04.....	34	7.25*
0.02.....	0	0.02.....	6	8.25*
0.01.....	0	0.01.....	0

* Where less than 50 per cent of the cysts were activated, the time given is for the total percentage of excystment.

amount present. Our results here confirm the first part of their statement concerning the concentration of the excystment substance. In our routine experiments we have varied the amount of the excystment medium from $\frac{1}{4}$ cc. to 1 cc. for a standard preparation, with no difference in results. However, to be able to say here that excystment is independent of the total amount of medium present, we would need to make further tests. Brown and Taylor (1938) found that yeast extract of a certain concentration was necessary to produce 100 per cent excystment of *Colpoda*.

b) *Effects of temperature on excystment.*—Variations in the temperature markedly affect the time of excystment in *Woodruffia*. The variation in the rate of excystment with temperature is shown in Figure 3.

The optimum temperature for excystment, as judged by excystment time, is 35° C. It was impossible to activate wet cysts at 15° C. Dry cysts, however, could occasionally be activated at this temperature, but the percentage of activation was low. Above 35° C. there is an increase in excystment time which is quite marked. As a temperature of 38° C.

² The dehydrated alfalfa leaf meal was obtained from the Denver Alfalfa M. and P. Co., Lexington, Nebraska.

is lethal (cf. Johnson and Evans, 1939), it is quite probable that cysts activated near this upper temperature limit are injured and that such injury prolongs the excystment period.

Thimann and Barker (1934) found the optimum temperature for excystment of *C. cucullus* to be 24° C. The optimum for *W. metabolica*, as shown here, is considerably higher.

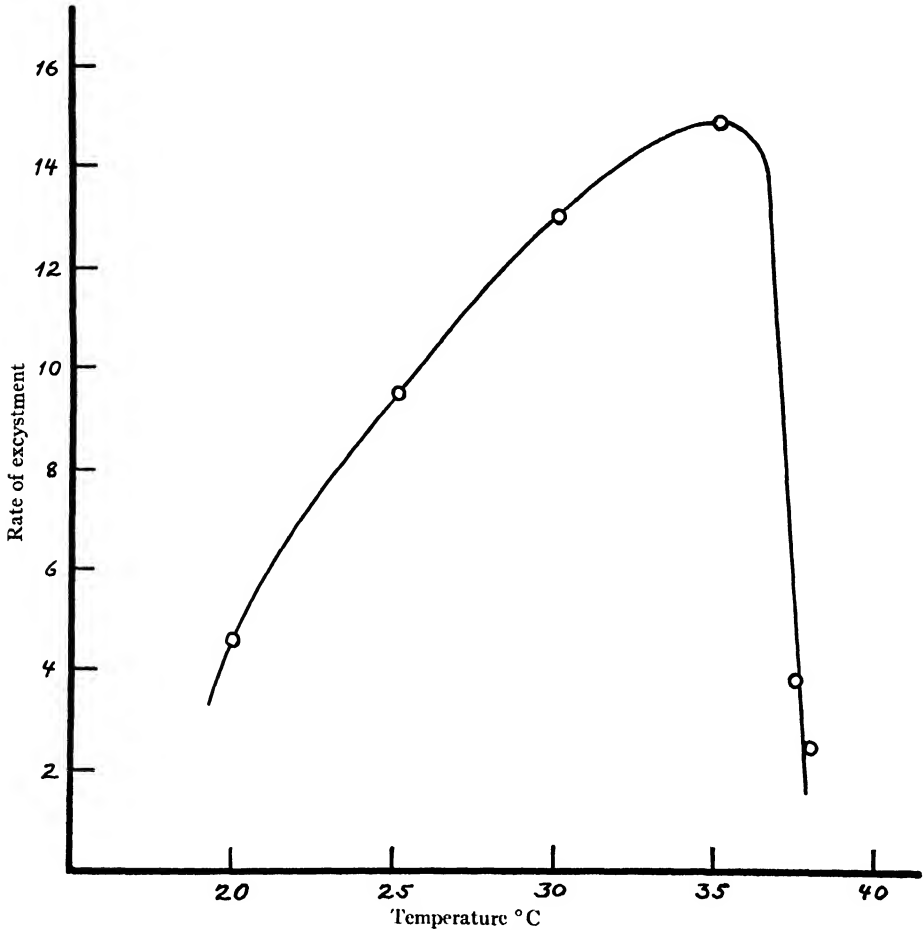


FIG. 3.—Effects of temperature on rate of excystment. The rate is expressed as the reciprocal of the time in hours times 100.

Various workers have found that resting cysts of a number of species of protozoans are quite resistant to extremes of temperature. Tests were made in these studies to determine the resistance of stable resting cysts of *Woodruffia* to extremes of temperature.

c) *pH of the excystment medium.*—Koffman (1924) and Darby (1929) have reported that changes in the H-ion concentration of the medium may induce both encystment and excystment in *Colpoda* and *Stylonychia*, respectively. Goodey (1913) and Bodine (1923) found that excystment of *Colpoda* occurred in an alkaline, neutral, or acid medium. Oth-

er workers, in recent investigations, have been unable to repeat the results of Koffman and of Darby, using the same and different organisms. To test the influence of H-ion concentration on the excystment of *Woodruffia*, three series of solutions (balanced medium, *Elodea* solution, and alfalfa solution), ranging in H-ion concentration from pH 5.0 through pH 9.0, were used on standard preparations of wet cysts at 25° and 30° C. The results are given in Table 2.

The results given here do not suggest that variations in the H-ion concentration of the medium, over a wide range, affect in any major way the excystment of *Woodruffia*. Excystment from the stable cyst does not occur at any H-ion concentration unless the excystment substance is present in sufficient concentration. The time required is slightly longer at pH 5.5. This concentration is near the acid death-point for free-swimming organisms, and Johnson and Evans (1939) found that cysts could not be formed in solutions more acid than pH 5.6. Readings taken at the end of these experiments showed that there had been some change in the pH of the alfalfa and *Elodea* media during the

TABLE 2
HOURS FOR 50 PER CENT EXCYSTMENT AT DIFFERENT H-ION CONCENTRATIONS

TEMPERATURE OF EXCYSTMENT MEDIUM	pH OF THE MEDIUM								
	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0
30° balanced salt*.....									
30° <i>Elodea</i>		8 25	7.5	7.5	7.5	7.5	7.5	7.5	7.25
30° alfalfa.....		8	7	6.5	6.5	6.5	6.5	6.75	6.5
25° <i>Elodea</i>		11	10 5	10 5	10 5	9 5	9.5	9.5	9

* The preparations started in the balanced salt solutions showed no signs of activation after 48 hours. Addition of the buffered alfalfa solutions to these preparations caused activation and excystment in the normal time.

tests; e.g., media started at pH 5.0 gave readings of pH 5.5 at the end, and those started at pH 9.0 gave readings of pH 8.5, on the average. The time of excystment over the range studied is essentially the same. In an earlier paper it was shown that encystment occurs with the same facility over the same range of H-ion concentrations. The H-ion concentration of the medium becomes a limiting factor for both encystment and excystment of stable cysts of *Woodruffia* at pH 5.5-5.6. Dried cysts responded throughout the same range of H-ion concentration as that reported in Table 2 for wet cysts.

d) *Effects of salt concentration of the medium on excystment.*—Thimann and Barker (1934) state that they avoided the use of salt concentrations greater than 0.02 M in their studies on *Colpoda* because that organism is osmotically very sensitive. It has been shown that free-swimming *Woodruffia*, when placed in solutions of the Osterhout medium varying in total salt concentration from 0.012 per cent to 1.2 per cent, inclusive, will form viable resting cysts (cf., Johnson and Evans, 1939). Solutions with a total salt content of 1.44 per cent were lethal to most of the organisms. In these experiments differences in the viability of *Woodruffia* cysts, prepared in different salt concentrations and activated in different salt concentrations, were tested. The criterion used was the time required for 50 per cent excystment.

Two series of tests were made. In one, standard cyst preparations were made in salt solutions of each of the following concentrations: 0.012, 0.024, 0.06, 0.12, 0.24, 0.6, and

1.2 per cent. When mature, these cysts were activated at 30° C. with the standard *Elodea* solution. In the other series standard cyst preparations were made in the usual way; and, when mature, these cysts were activated at 30° C. with *Elodea* solutions made in the following salt concentrations: 0.012, 0.024, 0.06, 0.12, 0.24, 0.6, and 1.2 per cent. Figure 4 shows the results graphically.

Normal cyst preparations were activated in the same time, using excystment media varying in total salt content from 0.012 to 0.24 per cent. In fifty times the X concentration a somewhat longer time was required for excystment, and in one hundred times the X concentration excystment was considerably delayed. These results parallel fairly closely the results previously obtained on the effects of salt concentration on encystment.

Cysts which formed in the different salt concentrations and which were activated with the standard *Elodea* solution responded in the same time in the different preparations,

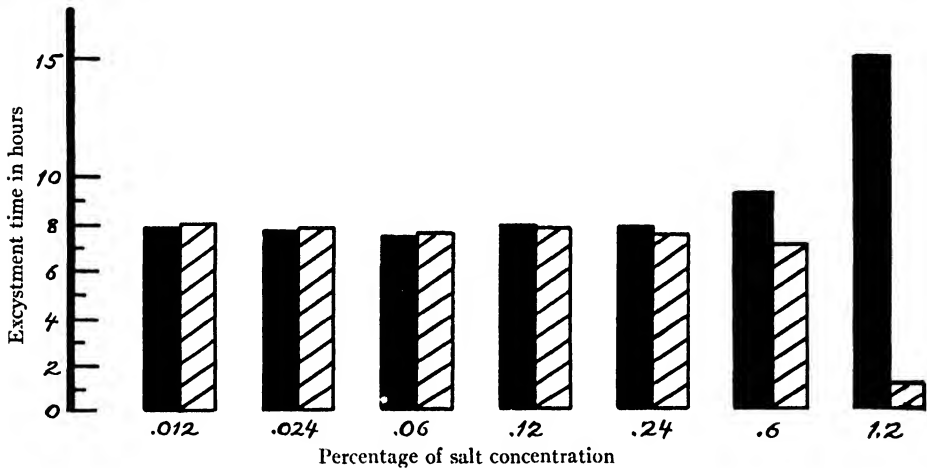


FIG. 4.— Effects of salt concentration on excystment. Solid bars, cysts formed in X salt concentration and activated at different concentrations. Cross-hatched, cysts formed in different salt concentrations and activated in standard *Elodea* solution.

with the exception of those formed in the 1.2 per cent salt concentration. These cysts were larger and had thinner cyst membranes than normal cysts, and excystment occurred in about 1 hour's time. This unusually rapid excystment was quite puzzling at the time, but it is now clear that they were the unstable type of cyst. A detailed discussion of unstable cysts formed in different salt solutions will be given in a later section.

These results show that normal resting cysts, as judged by the excystment time, may be formed and also may be activated, over a wide range of salt concentration of the medium. Weyer (1930) was able to obtain normal cysts of *Gastrostyla* over an even greater range of salt concentrations—from 0.006 to 2.0 per cent total salt concentration. In this study Weyer did not report the formation of cysts comparable to the unstable type reported here in the higher salt concentrations. In a number of different attempts it has been possible to induce the formation of normal *Woodruffia* cysts in the balanced salt medium with a total salt content as low as 0.003 per cent. Thus, the range in salt concentration of the medium in which this organism can form resting cysts is 0.003 per cent to 1.2 per cent, inclusive.

e) *Bacterized Elodea solution as an excystment medium.*—*Woodruffia* will form stable resting cysts in the *Elodea* solution and in other solutions which may be used as excystment media. The question arises: Do the bacteria which grow in such solutions in some way inactivate the substance or substances which cause excystment? To test this, some *Elodea* solution was bacterized with mixed bacteria from a hay infusion and then incubated at 25° C. for 3 weeks. At the end of this time the solution was filtered through a Chamberland filter to remove the bacteria. It was then used as excystment medium on standard cyst preparations. The excystment which resulted in five tests was normal both as to percentage of excystment and as to excystment time. In other words, excystment with this medium was quite similar to that obtained in control preparations in which sterile *Elodea* solution, prepared at the same time and incubated, was used.

If bacterial action does not alter the excysting potency of such solutions, why do cysts formed in such media remain in the encysted state over long periods? Thimann and Barker (1934) found that mixing excystment medium of *Colpoda* cultures with their ex-

TABLE 3
EFFECTS OF MOMENTARY EXPOSURE TO HIGH TEMPERATURES

DRY CYSTS		WET CYSTS	
Temperature (° C.)	Percentage of Excystment	Temperature (° C.)	Percentage of Excystment
100.....	100	45.....	87
110.....	97	48.....	85
120.....	100	51.....	86
125.....	92	54.....	88
130.....	0	57.....	0
135.....	0	60.....	0

cystment medium caused a reduced percentage of excystment. They say (p. 47): "Medium in which encystment has occurred in some way reduces the sensitivity of the organisms to the excystment stimulus. The action is upon the organisms themselves and not upon the excysting substance." The evidence presented here indicates that bacterial action does not inactivate the excystment substance. Further studies are needed to show just how the encystment medium changes the sensitivity of the organisms to the excystment stimulus.

f) *Resistance to extreme conditions.*—Bodine (1923) found that dried *Colpoda* cysts could withstand a temperature of 100° C. (higher temperatures were not tested) and that wet cysts, gradually heated, were viable after a momentary exposure at 55° C. Taylor and Strickland (1936) confirmed and extended this observation in their studies on *Colpoda*. They found that the upper critical temperature for wet cysts with momentary exposure was 51° C. and for dry cysts with momentary exposure was 121° C. Similar tests were made on wet and dry cysts of *Woodruffia*. The wet cysts were 4-day-old cysts; and the dried cysts, which had formed on Cellophane, were dried at room temperature for 2 weeks. Taylor and Strickland (1935) found that *Colpoda* cysts dried on Cellophane retained their viability longer than cysts dried on any other substrate. The results are given in Table 3.

Dishes of dried cysts were placed in an oven; and as soon as the temperature reached each interval to be tested, a dish was removed from the oven. From these results it appears that the upper lethal temperature for dried *Woodruffia* cysts is between 125° and 130° C. Dishes containing the wet cysts were placed in a water bath, and the liquid surrounding the cysts was constantly stirred with the bulb of a thermometer until the desired temperature was reached. The dishes were removed from the bath, and the cysts tested for excystment. The upper lethal temperature for wet *Woodruffia* cysts is between 54° and 57° C. These results are quite comparable to those obtained by other workers on the resistance of *Colpoda* cysts to high temperatures.

The question arises: When do such cysts begin to have this resistance to high temperatures? It has already been shown that 40° C. is lethal to the free-swimming organism. To test this matter, several standard cyst preparations were started at room temperature. As soon as the organisms had rounded up, one dish was placed in an incubator at 40° C. Following this, at intervals of 11, 19, 28, 35, 45, and 60 hours, other dishes were placed in the incubator at 40° C. In all the preparations, up to those transferred at the

TABLE 4
EFFECTS OF FREEZING ON STABLE CYSTS

Period of Freezing	Excystment Time	Percentage of Excystment
Control	7 5	98
24 hours	8 0	90
1 week	8.3	85
2 weeks	11 0	91
2 months	9 0*	20

* Excystment time refers to hours for 50 per cent excystment in each case except for those cysts frozen for 2 months. Here the excystment was only 20 per cent, and the time given is for that percentage

thirty-fifth hour, the organisms were still rotating quite rapidly within the cyst wall. After 4 days these cysts were removed and tested with the alfalfa solution for excystment. In the dishes transferred to 40° C. at the beginning and after 11, 19, and 28 hours, no excystment occurred. In all the other dishes excystment was over 90 per cent and in the normal time. These cysts, then, become resistant to a temperature which is lethal to the free-swimming organism after encysting at room temperature for about 35 hours.

Bodine (1923) found that cysts of *Colpoda* were viable after freezing. Taylor and Strickland (1936) obtained some excystment from preparations of *Colpoda* cysts which had been exposed to liquid-air temperature for 12½ days. The effects of freezing on *Woodruffia* cysts are given in Table 4. Dishes containing 4-day-old standard cyst preparations were placed in the ice chamber of an electric refrigerator. The medium surrounding the cysts was frozen in 2 hours. At the end of each time-interval to be tested, cyst preparations were removed to room temperature for 24 hours and then tested with *Elodea* solution for excystment.

The results given here are the averages of three preparations in each case except for those cysts frozen for 2 months. Freezing for 2 weeks has little effect on the viability of these cysts, but the excystment time was somewhat lengthened. After 2 months of freezing, however, in the one test made, the percentage of viable cysts was considerably

reduced. Bodine (1923) referred to the fact that similar protozoan cysts regularly undergo long periods of freezing in certain regions. So it seems probable that further testing might reveal that these cysts could withstand even longer periods of freezing, and, perhaps, with even higher percentages of viability than was obtained in this one test.

In addition to being able to withstand extremes of temperature, some protozoan cysts will remain viable for extremely long periods of time in the dried condition. Dawson and Hewitt (1931) report that *Colpoda* cysts could be activated after being in the dried state for 5 years and 4 months. Taylor, in this laboratory, has kept dried *Colpoda* cysts for an even longer time without complete loss of viability. Beers (1937) reviewed the literature on the viability of dried cysts of different protozoa over long periods of time. In his studies he found that, although *Didinium* cysts were not viable after drying, some cysts were still alive after 10 years in hay infusion. The indications are that *Woodruffia* cysts are comparable to *Colpoda* cysts in their ability to withstand drying over long periods. The effects of aging on viability, in the tests made to date, are given in Table 5. The dried preparations used here had encysted on Cellophane.

TABLE 5
EFFECTS OF AGING ON VIABILITY OF CYSTS

DRY CYSTS		WET CYSTS	
Age in Days	Percentage of Excystment	Age in Days	Percentage of Excystment
151.....	90
372.....	85	372.....	85
516.....	65

Standard 4-day-old preparations give from 95 to 100 per cent excystment. With aging, there was some falling-off in percentage of excystment, but after more than a year, 85 per cent excystment was obtained in both wet and dry cysts. Taylor and Strickland (1935) found that there was a gradual decrease in the viability of *Colpoda* cysts during the first 6 months after formation to about 75 per cent, and that the viability remained at this level for three more months. They also found that the time required for excystment was increased with the aging of the cysts. That is also true for *Woodruffia* cysts. Dry cysts, over a year old, required approximately three times as long for excystment as standard control preparations; and wet cysts, of the same age, required twice as long for excystment as control preparations.

The problem of whether protective cysts in a thoroughly dried condition carry on any metabolic activities is an interesting one. Darby (1929, p. 29) says, in a discussion of his methods of determining the viability of cysts of *Stylonychia*, that "the third [method] was by observing their respiration in the dry state by using Parker's method of CO₂ determination"; but he gives no further details of this test. To our knowledge, no one has yet reported a really critical test on this matter.

UNSTABLE CYSTS

"Unstable" resting cysts have already been defined as cysts which can be activated without the addition of some organic excystment substance. Cysts which behave in this

way were first encountered in our studies when *Woodruffia* were induced to encyst and excyst seven successive times without feeding. After the third excystment, such cysts could be activated by merely changing the salt solution at room temperature (cf. Johnson and Evans, 1939). This behavior, although not explained, was attributed to the small size and starved condition of such cysts. In a later study, the original purpose of which was to prepare cysts at 30° C. and determine whether there was any difference in the excystment time of such cysts and the standard preparations formed at 25° C., cysts were obtained which could be activated by changing the salt solution. Four dishes, each containing 30 *Woodruffia*, were placed in an incubator at 30° C. After 4 days two of these dishes were removed to test for excystment. Considerable evaporation had occurred in these preparations, probably because insufficient water had been added to the Petri dishes for the higher temperature. The alfalfa solution was added to one dish, and fresh balanced salt solution was added to the other as a control. In the earlier experiments on 25° cysts salt controls were run for numerous trials with no excystment. In both of these preparations 50 per cent excystment occurred in much less than the average time required at 30° C. The other two preparations were then tested in the same way. An examination of these cysts showed that from 75 to 80 per cent of the cysts in each dish were large and thin-walled. These thin-walled cysts excysted in approximately 75 minutes in each dish. The thick-walled cysts never excysted in the control dish, but in the dish to which alfalfa solution had been added they excysted in from 6½ to 7 hours.

Our efforts were then directed to finding the factors which cause the formation of such cysts.

a) *The formation of unstable cysts.*—In the first attempts to obtain such cysts again, poor results were obtained. Out of 56 trials at 30° C., in which the organisms were prepared in the same way in which all standard cyst preparations had been prepared, unstable cysts were obtained in only 16 of the trials, with an average formation for the 56 trials of 2 per cent. To prevent evaporation of the solutions containing the cysts in these tests, precautions were taken by placing the Petri dishes in large moist chambers. In this way a double insurance against evaporation of the culture medium was provided.

The microscopic appearance of the unstable cysts suggests that there are fewer granules in the protoplasm of such cysts than in stable cysts, or that the granules in the unstable cysts are more diffuse. It was thought that the use of organisms containing fewer granules might produce the unstable type of cyst in higher percentages. Such animals can be obtained by delaying encystment. Delay in encystment may be caused by agitating free-swimming organisms when they form clumps to start encystment. By carefully watching preparations containing organisms ready for encystment, sucking up into a pipette the clumps which formed, and then ejecting them back into the culture dish, encystment was delayed from 4 to 5 hours. Such preparations were then allowed to encyst at 30° C. Out of 28 trials, unstable cysts were formed in only 7 of them, and in these instances very few such cysts were formed. The average percentage for all of these trials was 0.7 per cent. The stable cysts formed appeared to have thicker cyst membranes than usual. So, instead of facilitating the formation of unstable cysts, this treatment hindered it.

One preparation of cysts formed at 30° C., in which the *Woodruffia* used had been feeding on paramecia in a semistarved condition (these paramecia had been washed and crowded in the balanced salt solution for a week without food), had 20 per cent of the cysts of the unstable type. Thirty-six similar tests were then made, and in 23 of them un-

stable cysts were formed. The percentages of unstable cysts formed in these tests ranged from zero to 62 per cent, with an average for all of the trials of 9.6 per cent.

In another series of tests 5X salt concentration was used for the encystment medium. Out of 20 trials, unstable cysts were formed in 17 of them, with an average percentage for all of the trials of 18.7 per cent. These two series of tests show that both the use of organisms which had fed on semistarved paramecia and the use of encystment medium with a higher salt concentration increased the formation of unstable cysts.

In all of the tests referred to above, the preparations were made by starting 30 *Woodruffia* in $\frac{1}{2}$ cc. of encystment medium. Other tests were made at 30° C. in which 300-400 *Woodruffia* were allowed to encyst in 1/20 cc. of medium. The *Woodruffia* for these tests were concentrated by centrifuging. To prevent evaporation in these cultures, large cover glasses were sealed over the depressions of the Columbia culture dishes. In 11 such tests, in which the *Woodruffia* used had been feeding on well-fed paramecia, 14.9 per cent of the cysts formed were of the unstable type. In 7 other tests, using *Woodruffia* which had been feeding on semistarved paramecia, the average formation of unstable cysts was 40.9 per cent. These results indicate that crowding may be a factor in causing the formation of such cysts and that the effectiveness of crowding in this connection is increased when coupled with the use of organisms which have been existing on a qualitatively deficient diet.

In only a few of the tests described above did the percentage of unstable cysts formed equal that of the four preparations in which this type of cyst was first noticed. It seemed desirable to be able to induce the formation of such cysts more consistently and in higher percentages. The fact that the smallest percentages of unstable cysts obtained in these tests at 30° C. were obtained in the tests where encystment was delayed, together with the variations obtained in the other tests, suggested that a disturbance of the organism in some critical period just prior to encystment might affect the ensuing encystment process. It is altogether possible that the organisms isolated for the tests were not always in exactly the same state with reference to encystment. To obtain cyst preparations without any disturbance of the organisms just prior to encystment, a series of experiments were run which will be referred to as "completion cultures." In these experiments paramecia were washed twice by centrifuging, and $\frac{1}{2}$ cc. suspensions of such paramecia (averaging 2,000-3,000 per cubic centimeter) were placed in culture dishes with one washed *Woodruffia*. Such cultures were then incubated at 25° and 30° C. for 9 and 7 days, respectively, and were then examined and tested for unstable cysts. The number of cysts formed in these preparations varied from 1,000 to 1,500. At 25° C. the *Woodruffia* required about 5 days to eat all of the paramecia and begin encystment; at 30° C. they depleted the food and started encystment in 3 days. The average results of these experiments are given in Table 6.

The differences between well-fed and semistarved food organisms have already been described. The results obtained here were consistent, and the percentages of unstable cysts much higher than those in the other tests. It is evident from these experiments that unstable cysts may form at 25° C. under certain conditions, although they are not formed in uncrowded preparations in the X salt concentration at this temperature. The effects of using *Woodruffia* which had fed on semistarved paramecia was quite marked in these experiments. Also, consistently higher percentages of unstable cysts were formed at 30° C. than at 25° C. This difference at the higher temperature may be due in part to some effect of the higher temperature on the food organisms over a period of days and

also to some difference in evaporation of the culture medium at the higher temperature. That evaporation has an effect was again evidenced in these experiments in two dishes not placed in the second moist chamber. In these two preparations formed at 30° C., in which the food of the *Woodruffia* prior to encystment had been well-fed paramecia, 100 per cent formation of unstable cysts occurred. However, in these two cultures evaporation had taken place almost to the point of dryness. These two preparations were not included in the results given in Table 6.

One series of six completion cultures was run at 25° C. in which 5X salt solution was used as the encystment medium. In these tests the *Woodruffia* which had been feeding on well-fed paramecia formed 45 per cent of the total cysts of the unstable type, while the *Woodruffia* which had been feeding on semistarved paramecia formed 96 per cent unstable cysts. These percentages are higher than those obtained in similar cultures at 25° C. using the standard salt solution. Other tests, using still higher salt concentrations, indicated that the percentage of unstable cysts could be raised by increasing the salt

TABLE 6
FORMATION OF UNSTABLE CYSTS IN COMPLETION CULTURES

	TEMPERATURE			
	30° C.		25° C.	
	Well-fed Food Organisms	Semistarved Food Organisms	Well fed Food Organisms	Semistarved Food Organisms
Trials	15	10	16	7
Average percentage of formation . . .	76.6	94.4	30.6	53.9

concentration of the medium. It was previously shown that *Woodruffia* forms viable cysts in salt concentrations up to 1.2 per cent. Six different experiments were conducted at 25° C., in each of which 30 *Woodruffia* were allowed to encyst in 1 cc. of balanced salt solution of each of the following concentrations: 0.012, 0.12, 0.6, 0.9, and 1.2 per cent. The results obtained in these experiments were quite consistent, and the averages are given in graphic form in Figure 5.

High percentages of unstable cysts were obtained in the experiments in which only one factor was varied—the salt concentration of the encystment medium. In the other tests evidence was obtained which indicates that crowding of the organisms, feeding the organisms on semistarved paramecia prior to encystment, and increasing the temperature may induce the formation of unstable cysts in varying percentages. In the completion cultures at 30° C. where all of these factors were involved, 94 per cent of the cysts formed were of the unstable type; yet in uncrowded preparations in 100X salt concentration at 25° C., 91 per cent of the cysts formed were of this type. Also, 100 per cent formation of the unstable cysts occurred in two preparations in which evaporation, almost to dryness, occurred. Further studies are being made to determine whether such cysts can be formed under other conditions. At present it appears that more than one factor, either independently or cumulatively, may operate in causing the formation of

unstable cysts. It is conceivable that all of the factors dealt with here might be operative in nature.

b) *Methods of activating unstable cysts.*—Reference to the fact that these cysts may be induced to excyst merely by changing the salt solution on them has already been made. Triple glass-distilled water was just as effective. In all cases in which the encystment medium was replaced by fresh salt solution or distilled water, activation of the unstable cysts was approximately 100 per cent. In several of the preparations some of the activated organisms, although they were able to break out of the mesocyst, were trapped in the ectocyst. In such cases the mesocyst appeared folded against the ectocyst. After a time these organisms would re-encyst. Only rarely are organisms in stable cysts trapped in

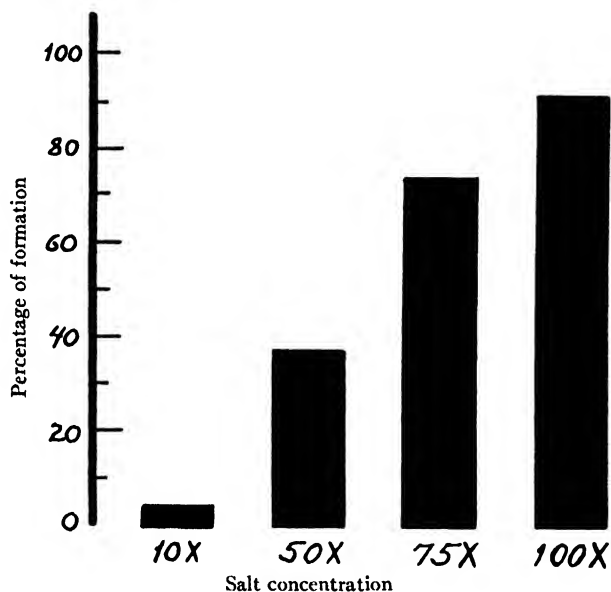


FIG. 5.—Formation of unstable cysts with increased salt concentration

the ectocyst—after activation. In two cases organisms trapped in the ectocyst of stable cysts have been observed to divide and then to re-encyst.

By accident two preparations of unstable cysts were left on the laboratory table without changing the salt solution when they were removed from the 30° incubator for the excystment test. When they were examined 2 hours later, all the unstable cysts had excysted. Numerous other trials were made, removing preparations of the cysts from the 30° incubator to a temperature of 20° C. and also to room temperature (23°–24° C.) without changing the medium on the cysts. In all such tests the drop in temperature was just as effective in inducing excystment as changing the medium on the cysts. Here is an instance where a process of activation may be induced by a lowering of the temperature, which is unusual. Precisely what is involved here is not clear, and further studies are being made on the nature of this activation. That a very delicate balance exists between such cysts and their environment is obvious because, by either replacing the encystment medium with the inorganic salt solution or with distilled water or by simply lowering the temperature, they can be activated.

The excystment time of unstable cysts is quite short, compared to that of stable cysts. Unstable cysts formed at 30° C. and excysted at room temperature require about 1¼ hours for the process, while stable cysts under the same conditions require 12–15 hours. Many of the unstable cysts will excyst in 45 minutes. In every case where such cysts were examined under a compound microscope just after they had been changed to fresh salt solution, some cyclosis in the protoplasm was noticed, and in a short time the vacuole or vacuoles present began to function.

c) *Effects of aging and extreme conditions on unstable cysts.*—A series of completion cultures were started at the same time at 30° C. to use in weekly tests on the viability of unstable cysts. Several such tests have been made—the first, 2 weeks after the start of the cultures; and the others, weekly thereafter. Approximately 100 per cent excystment of the unstable cysts occurred in each test. This indicates that there is no change in the viability of these cysts for a period of more than 2 months. Additional tests on the effects of aging will be made.

Freezing for 24 hours did not affect the viability of unstable cysts. After thawing at room temperature all such cysts were activated by changing the salt solution on them. Luyet and Gehenio (1938) found that ice crystals did not form in the cells of moss leaves when their water content was 30 per cent or lower, and they imply that ice crystals will not form in cells with such a low water content. It seems probable that the unstable cysts of *Woodruffia* have a water content below the level where ice crystals will form, although they quite obviously have a higher water content than the stable *Woodruffia* cysts.

On the other hand, thorough drying is lethal to the unstable cysts. The encystment medium on several preparations was allowed to evaporate gradually over a period of 2 days in the 30° incubator. The first excystment test was made just as soon as all visible traces of moisture were gone. In this test 2 per cent of the cysts were activated. Ten other tests were made on successive days with no activation. Microscopic examination of these thoroughly dried cysts showed that the thin cyst walls were quite wrinkled and collapsed in appearance. Death, in these cysts, probably resulted from pressure on the protoplasts due to the distortion of the cyst walls during drying. The thick walls of stable cysts are not so affected by drying.

DISCUSSION

The results presented here on the effects of environmental factors on stable cysts of *W. metabolica* are similar in many respects to the results obtained in this laboratory by Taylor and his associates in carefully made experiments on cysts of *C. duodenaria*. Stable cysts of *Woodruffia* behave much like cysts of *Colpoda* in the following regards: (1) the presence in the medium of some organic substance above a certain concentration is necessary for excystment, (2) the time required for excystment is dependent on temperature and, within certain limits, the excystment time is shorter the higher the temperature, (3) over a wide range the hydrogen ion concentration of the medium does not affect excystment, (4) freezing for long periods does not lower greatly the percentage of viable cysts, and (5) such cysts can withstand thorough drying for long periods of time.

The discovery of a second type of protective cyst, the unstable cyst, in *Woodruffia*, which forms under certain conditions almost 100 per cent and which differs from the stable type both morphologically (in that the ectocyst is much thinner, the protoplast larger, the granules more diffuse, and a vacuole present) and physiologically (in that no

organic substances are required for excystment and in other ways), raises a number of interesting questions.

As mentioned above, it is not clear at the present time whether several factors working independently operate to produce the unstable cysts or whether there is some underlying single factor which causes the formation of such cysts. The facts that crowding, increasing the temperature, and increasing the salt concentration of the medium tend to increase the percentage of such cysts and that a mere lowering of the temperature will activate them suggest that changes in oxygen tension may be involved both in the formation and in the activation of these unstable cysts. Tests are now being made to determine if such relationships exist.

It has been recognized (Taylor, 1935) that a more or less thorough dedifferentiation of the protoplasm occurs during encystment. Garnjobst (1937) has reviewed the literature on this subject, pointing out that differences in degree of dedifferentiation occur both within a single species and between species. While no studies have been made on the differences between the stable and unstable protective cysts of *Woodruffia* as regards dedifferentiation, certain things are suggested. The presence of a vacuole in the unstable cysts is indicative of less dedifferentiation than that in stable cysts. Cyclosis or motion in the protoplast of unstable cysts is noticeable when examined under a microscope immediately after their removal from a preparation. Definite rotation within the cyst wall occurs after several minutes, and the whole process of excystment is often completed in 45 minutes. In contrast with this, stable cysts require about 6 hours for excystment under the most favorable conditions. These differences also suggest that the unstable cysts are in a less dedifferentiated state than stable cysts. The factor or factors which induce the formation of unstable cysts seem to inhibit the secretory processes involved in the formation of the ectocyst, and it appears likely that the process of resorption and dedifferentiation is also inhibited, or at least in part. Garnjobst (1937, p. 369) found that, "of the two hypotrichs under consideration (*Stylonethes* and *Euplotes*), the cysts which withstand drying show a greater degree of dedifferentiation." Drying is lethal to the unstable *Woodruffia* cysts, while the stable cysts are able to withstand drying for long periods. The question of the degree of dedifferentiation in the two types of *Woodruffia* cysts will be the subject of a future investigation.

Schewiakoff (1928) describes degrees of encystment in *Euplotes harpa*. He was able to induce various cystic forms in this organism, depending on the rate of evaporation of the medium. If the process was rapid, the organisms merely came to rest without secreting a wall. By less rapid evaporation the organisms rounded up, but only a part of them formed a thin cyst membrane. By slow evaporation, occupying 2-3 days, the organisms always formed a rigid cyst wall. All these stages could be activated. Garnjobst (1937) refers to an unidentified species of *Euplotes* which would, during evaporation of the medium, become motionless and remain alive in a stiffened condition for several days without secreting a cyst wall. The unstable and stable protective cysts of *Woodruffia* described here may, in a certain sense, be regarded as degrees of encystment. They differ in degree in the thickness of the ectocyst; and, on the basis of the evidence now at hand, they probably differ in the degree of dedifferentiation and in the amount of water present in the protoplasm. However, the idea of degrees of encystment in *Woodruffia* does not imply that the unstable type of cyst may gradually become the stable type. Close observations for over a month have not shown any such transformations.

In comparing results obtained in cystment studies on different forms, it is important to know whether the cysts can withstand drying and whether they require the presence of some organic excystment substance in the excystment medium, as well as the degree of dedifferentiation of the cysts. It is also important to know whether more than one type of cyst, with reference to these conditions, can be formed by different species. While no other detailed studies have been made on the formation of more than one type of protective cyst, other than the study of Schewiakoff referred to above, a partial review of the literature on protozoan cystment suggests that more than one type of protective cyst may be formed by other species.

In certain instances it is clear that the cysts described required some organic excystment substance for activation. Weyer (1930) attributed excystment of *Gastrostyla* to the products of bacterial action in the excystment medium. Barker and Taylor (1933) and Taylor and Strickland (1938) state that an extract of plant or animal cells is necessary for the excystment of *Colpoda*. Haagen-Smit and Thimann (1938) attributed the excysting potency of hay infusion on *Colpoda* cysts to the salts of simple organic acids present in it. Garnjobst (1937) found that the protective cysts of *Stylonychia*, which could withstand drying, required the addition of a wheat-infusion medium for excystment.

In other instances investigators have described the occurrence of excystment simply upon the addition of water. Bodine (1923) reported that excystment in *Colpoda* occurred upon the addition either of hay infusion or of tap water. Moore (1924) found that spontaneous excystment occurred in *Spathidium*. Such cysts were not viable after drying. Garnjobst (1928 and 1937) indicates that protective cysts of *Euplotes taylori* could be induced to excyst by adding either tap or distilled water. These cysts would not withstand drying. Darby (1929) caused cysts of *Stylonychia* to excyst both by adding hay infusion and by adding spring water to the preparations. In the studies of Beers (1930 and 1935) on *Didinium* it is not clear whether the cysts required an organic substrate for excystment. In this work the cysts were always transferred from either spring water or hay medium to fresh hay medium for excystment. Although it can be argued that in these cases where the addition of water caused excystment there was sufficient organic matter in the water to produce it, our experiences with the two types of protective cysts in *Woodruffia* suggest an alternative explanation—that these cysts did not require any organic excystment substance for activation.

Certain results obtained in different studies on *Colpoda* cysts suggest that an unstable type of cyst may be formed by this organism. Barker and Taylor (1933, p. 129) reported that "replacement of the encystment medium by fresh balanced medium—the same in which the organisms originally encysted—may or may not induce excystment. It occasionally does so." Thimann and Barker (1934) found that, when they diluted a preparation of *Colpoda* cysts in 0.2 cc. of balanced medium with 4.8 cc. of sterile tap water, they obtained an average excystment of 72 per cent in three such trials. They attributed this result to a dilution of the encystment medium which makes cysts less sensitive to the excystment stimulus and to the presence of traces of organic matter in the sterile tap water, which gave it a weak excysting potency. The significance of the latter suggestion is not clear in view of their other finding that the percentage of excystment depends upon the concentration and not upon the total amount of excysting material present. Haagen-Smit and Thimann (1938) experienced occasional small percentages of excystment of *Colpoda* cysts in water alone. They attributed these results to the influence

of dust from the air and thereafter conducted their experiments in a constant temperature room of 24° C. with a high humidity. Such excystment in water also suggests the possibility that in these instances they were dealing with cysts comparable in behavior to the unstable type we have described here. In a recent study Taylor and Strickland (1938) found that sterile *Colpoda* isolated in a small drop of sterile balanced medium would encyst and then excyst spontaneously. Such behavior has something in common with that of the unstable cysts described here, i.e., excystment is not dependent upon some organic substance. However, the relationships, if there are any, between such *Colpoda* cysts and the unstable *Woodruffia* cysts are not clear. The very careful method used in making preparations for the standard tests made on *Colpoda* cysts by Taylor and his students in recent years would eliminate from their preparations cysts comparable to the unstable ones found in *Woodruffia*, if any such are formed. Brown and Taylor (1938, p. 477) describe their method of preparing cysts in these words: "The cyst-Cellophane preparation is cut in strips which are hung in continuously flowing balanced medium (Osterhout's, 0.012 per cent total salt) at 20° C. Under these conditions the same preparation yields uniform results for a period of many months."

It is difficult to make comparisons of results obtained in different investigations. The inferences made here are in the form of suggestions—suggestions that comparative studies should be made on a number of different cyst-forming protozoans to determine whether more than one type of protective cyst can be induced in other protozoans under reproducible conditions. If adequate generalized conclusions are to be made about protective cysts of different species of protozoa as regards their viability upon drying, their requirement of an organic excystment substance, their state of dedifferentiation, and as regards other matters of comparison, it is necessary that we know whether more than one type of protective cyst may be formed in each species, so that such conclusions will be based only on comparisons of cysts which are similar in nature. On the basis of the results presented here, it is suggested that possible criteria for distinguishing between two types of protective cysts in other organisms, purely aside from structural differences, are the ability to withstand drying and the need of plant or animal extracts in the excystment medium.

SUMMARY

Two types of protective cysts in *W. metabolica* are described. These cysts differ both morphologically and physiologically and are referred to as "stable" and "unstable" cysts. Encystment, leading to the formation of either type of cyst, is dependent upon the absence of food under the conditions of these experiments.

Excystment of the thick-walled stable cysts is dependent upon the presence of plant extracts in certain concentration in the excystment medium. The rate of excystment of stable cysts is a function of temperature, within certain limits. Excystment of such cysts is not materially affected by variations in the H-ion concentration of the excystment medium over a wide range (pH 5.5–9.0). Stable cysts will excyst in solutions with salt concentrations ranging from 0.012 to 1.2 per cent. The potency of plant extracts used for excystment is not changed by bacterial action over a period of 3 weeks. Stable cysts can withstand freezing and drying for long periods. These cysts, in a thoroughly dried condition, are killed at temperatures between 125° and 130° C., while in the wet state they are killed at temperatures between 54° and 57° C., with momentary exposures.

Crowding, the use of organisms which had fed on semistarved paramecia prior to encystment, and increasing the temperature and increasing the salt concentration of the

medium—all influenced the formation of the thin-walled unstable cysts. Excystment of these cysts could be induced by changing the balanced salt solution, by replacing the old salt solution with distilled water, or by merely lowering the temperature of the preparation from 30° to 20° C. or to room temperature. The excystment time of unstable cysts is much shorter than that of stable cysts. Unstable cysts can withstand freezing for 24 hours but are killed by thorough drying. A vacuole is always present in unstable cysts, which, together with other differences, suggests that the unstable cysts are in a less differentiated state than the stable cysts.

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UNSOLVED PROBLEMS OF GENERAL BIOLOGY¹

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In 1895 Delage² wrote as follows:

La Biologie générale comprend, malgré son nom, un grand nombre de problèmes particuliers. La division cellulaire, le mouvement du protoplasma, la formation des espèces, ne sont pas certes des questions mesquines; cependant on peut entreprendre de les résoudre sans connaître la constitution intime de la substance organisée et ses modes d'action. Ce sont, malgré leur ampleur, des questions particulières. La Biologie renferme quelque chose de plus général, c'est ce qui concerne l'essence même de la vie, comment elle commence, se continue et se transmet.

Il n'y a pas deux manières d'attaquer le problème de la vie pour en trouver une solution positive. La vie résulte évidemment des propriétés de la matière vivante et ces propriétés sont le résultat de sa constitution chimique. Or là, nous sommes radicalement arrêtés. La chimie nous enseigne la composition élémentaire du protoplasma; elle nous fait connaître un certain nombre des substances complexes qui entrent dans la constitution de ses différentes parties; l'histologie nous montre en lui des organes minuscules fort complexes, associés pour former la cellule; mais entre les molécules, que le chimiste ne fait que peser ou compter, et ces organes déjà si compliqués, il y a une lacune immense. Quel arrangement prennent les molécules? Quelles associations forment-elles pour engendrer la substance vivante?

Notre ignorance est absolue sur ce point.

There is no doubt that, at the time of this writing, this statement correctly expressed the profundity of our ignorance concerning the chemical structure of living protoplasm. The recognition that certain elements, extracted from living substance—such as carbon,

¹ This is the second of an invited series of synthesizing papers. Others, which will appear in future numbers, include the following:

J. H. Bodine, Exact title to be announced later—some phase of cellular physiology.

Alan Boyden, "Serology and Animal Relationship."

L. V. Heilbrunn, "The Relation of Protoplasm to the Calcium Ion."

R. S. Lillie, "Artificial Parthenogenesis in Relation to the Problems concerning the Initiation of Development."

B. H. Willier, "Embryonic Organization in Presomite Chick Blastoderms."

² Y. Delage, *La Structure du protoplasma et les théories sur l'hérédité et les grands problèmes de la biologie générale* (Paris: Reinwald, 1895).

hydrogen, oxygen, and nitrogen—enter into the structure of carbohydrates, lipins, and proteins did not appreciably dissipate this ignorance. Nor could it have done so, since the living substance is a complex comprising not only these organic compounds but also water and electrolytes. The structure and behavior of living substance are derived from the reactions embracing elements acting singly or with each other and with the organic compounds and water. Upon these combinations no resolution into elements can throw any light, since an analysis of a mineral water into constituent elements does not tell us what compounds of them are present. Protoplasm, far more complex than a mineral water, resolved into constituent elements, or into compounds even, is no longer protoplasm.

After forty-five years this gap in our knowledge persists. Despite the undeniably rich contributions of biochemistry, which have increased our knowledge of the organic compounds, especially that of the proteins which enter into the structure of the cell—and despite the elegant studies of the effects of electrolytes on vital behavior, the vast array of researches on the permeability of protoplasm to water and to solutions, and the formidable attack on other cell problems by biophysicists—we are far from understanding, on the basis of protoplasmic structure broken down into elements, protoplasm as a living self-perpetuating system. Between the supposed beginning, the elements, and the end-point, the cell, we have, in default of exact knowledge, only vague conceptions. No less has orthodox biology, which deals with the morphology of the cell as such, failed to grasp the meaning of this gap in our knowledge.

This ignorance being admitted, the above-quoted statement is of an incontestable fact. It may be otherwise with Delage's judgment that the grand problems of general biology are pendant to the problem of the structure of protoplasm. Some, perhaps many workers, may disagree with it. To me, however, it seems even more apparent today than it was in Delage's time that the only approach to the elucidation of the great riddles of what I denominate "general biology" is by way of knowledge of the structure of protoplasm. The successes—or, more correctly, the "failures"—of theories of general application emanating from particular studies on one cell component, e.g., the chromosome, are unmistakable signs that we need recourse to a practicable principle which envisages life-phenomena in the entire confines where life resides.

The term "general" connotes, for me, the quality of commonness. Thus, "general embryology of animals," for example, accurately speaking, should embrace only those phenomena common to all animal eggs, such as maturation of the egg, its cleavage (early or late), and the delimitation of ectoderm and endoderm—the sole exhibitions commonly displayed by animal eggs whereby they transform themselves into embryonic primordia. Similarly, the term "general physiology" ought to include only phenomena common to both animals and plants. But many a most excellent textbook on this subject discusses nerve and brain, blood, blood vessel and heart, and muscle of all kinds. Since no plant possesses any one of these and many animals lack one or all of them, we may explain this widespread practice as legitimate for teaching purposes. In a more strictly scientific essay one cannot be too exact—least of all in a science in which definition means so much can one use terms loosely. In this wise I consider the term "general biology" to include all those phenomena revealed to us by both animals and plants, and only those phenomena. How living things come to be and how they maintain themselves and transmit themselves constitute the chief problems common to all animals and plants; they are problems of general biology.

Now the visible or tangible expressions of livingness, i.e., the phenomena of the life-state, do not, so far as we know, exist apart from that material configuration which we denominate "protoplasm." Therefore, when one agrees that "matter as such produces nothing, changes nothing, does nothing"³—although the "as such" saves us, if it does not beg the question—one does not escape the necessity of seeking to know what the substance called "protoplasm" is, and how it acts and is acted upon as center and object of force and as seat of energy. The very fact, as stated, that "when we deal with matter in the concrete force does not, strictly speaking, enter into the question, for force, unlike matter, has no independent objective existence"⁴ demands of us a knowledge of the concrete substance "protoplasm." While it would be a mistake to exaggerate this concreteness, especially by too great an emphasis on static qualities, it would be an even greater error to supplant the concrete system by a subjective and symbolic term. On what, in what, and with what, force acts is the major problem for him who deals with life as something tangible and objective.

Either we conceive of life in each species of animal and plant as *sui generis*, or we associate the all-pervading life-condition with a common factor. The former point of view renders futile any attempt to reduce life-processes to a common basis on which we may hope to understand those phenomena that experience tells us separate the living from the nonliving. The latter, however, urges us to seek in all life-exhibiting states for common properties, a common substratum in which reside plant and animal characteristics, and, in the members of each of these kingdoms, their specific qualities. It is from this latter point of view that I envisage the problems of general biology. Living things have a common denominator, a basic structure, in which life resides and from which vital manifestations arise. Then life, as force, principle, or what you will, is associated with an organization, a discrete pattern which is common to all living things, be it plant or animal. The one basic feature common to all living things whose phenomena, tangible manifestations, we may apprehend, I conceive resident in protoplasm. As that which sets off the life-state from nonlife, protoplasm is peculiar to living things. We need to know what this substance is without which life does not reveal itself to us, in order to approach an understanding of the grand problems of general biology. These thus center around the structure of protoplasm, conceived not merely as static "matter as such" but as that which by its very impermanence gives life, or, rather, that succession of states which in their ensemble give livingness.

Here I would enter an exception: to amend the foregoing statement of Delage to read "protoplasmic system" instead of "cell." With no wish whatsoever to appear pedantic, I must, nevertheless, in a discussion of the ground-postulate of general biology insist upon clear definition. "Protoplasmic system" and "cell" not being synonymous, I reserve the term "cell" for that protoplasmic system which is composed of cytoplasm and discrete nucleus. This definition recognizes that some protoplasmic systems exist which do not contain a discrete nucleus, although they may possess nuclear matter in a granular or diffuse state. The question of the existence of protoplasmic systems devoid of nuclear stuff remains open. In view of the existence of the various types of protoplasmic systems we may say that a discrete nucleus—or several nuclei, as in syncytia—is not a *sine qua non* of living protoplasm. Strictly speaking, therefore, the structure of

³ D'Arcy Wentworth Thompson, *On Growth and Form* (Cambridge, 1917), p. 14.

⁴ *Ibid.*, p. 11.

nucleated protoplasm does not come within the domain of general biology, as we have defined "general biology" above. This is more than a point for special pleading. Since there are bacteria, algae, and perhaps protozoa, without discrete nuclei, although diffuse or granular nuclear material may be present in them, we cannot dismiss such forms of protoplasmic systems as "exceptions." They are living things and display their life-activities within the framework of protoplasmic structure. In the following pages I keep in mind that biologists recognize the existence of nonnucleated living substance, although I discuss, in the main, the protoplasmic system possessed of a discrete nucleus. This exposition concerning the structure of the protoplasmic system "cell" may serve as an orientation for more profound study of other protoplasmic systems which so far have not received the attention they merit in the interest of furthering knowledge of general biology.

Study of the living cell may be pursued from any one of three points of view: the biological, including the morphology and the physiology of form and form changes; the chemical, involving composition and chemical changes; or the physical. No one of the three is exclusive of the other; each is an auxiliary of the others. But, given the fact that life is ever associated with a kind of organization, is never revealed apart from this organization peculiar only to living things, the first duty of the biologist is the recognition of the concreteness of the life-state, its existence as objective, as an entity. Certainly—in the case of living cells, at least—it is as a tangible mass of substance that they attract attention; we may advance farther in our investigation of these forms of the protoplasmic system than of those that are nonvisible and intangible. Therefore, no matter what the special field of study, the first problem is to know fully the living cell from the biological point of view.

I

As defined above, the cell is that form of protoplasmic system which comprises cytoplasm and a discrete nucleus. Although in this exposition I limit myself to the discussion of animal cells, emphasizing especially egg cells, what I set forth none the less holds true also for plant cells.

Of the living cell, the extra-nuclear component is generally the more important quantitatively. In the longest nerve cells of the human body and in egg cells, the amount of nuclear stuff is less than the cytoplasmic. In spermatozoa the fact that nuclear volume is greater than cytoplasmic perhaps runs with the great degree of specialization that limits so sharply their functions and viability. However, not the whole content of the cell external to the nucleus is living cytoplasm. The first question to be answered, therefore, is: What in the perinuclear area of the cell is living substance par excellence?

The whole of any protoplasmic system, I hold, is a continuous hyaline menstruum, the ground-substance. According to this conception, in the case of cells the ground-substance pervades both nucleus and cytoplasm. It is this menstruum alone that I consider as living substance.

With respect to the cytoplasm, arguments may be adduced from both descriptive and experimental biology to support the proposition that only its ground-substance is living.

In tissue cells, such as secreting cells, visible granules appear and disappear according to states of cellular activity. Their disappearance may also result from experimentally induced stimulation. In fatigued cells or in cells suffering from lack of food, granules are less numerous. Since fat is transported across the intestinal wall, oil globules appear

most abundantly in the cells of the intestine. Thus, certain types of formed bodies in cells arise as products of metabolism or are food materials. Others are undoubtedly merely effete material. No one would denominate, as "living," particles of carmine, of ink, or of iron filings introduced into a *Paramecium*. So, other inert materials entering or leaving a cell, as well as those due to secretion and excretion, are not necessarily living because they are discerned within the cell boundary.

An animal egg cell at the normal moment of its fertilization is richly freighted with various cytoplasmic inclusions, such as oil, yolk, mitochondria, pigment granules, etc. But it did not possess all these at the time when it began its history as a primordial germ cell. Indeed, at the outset it is often difficult to distinguish, among primordial germ cells, which is to be egg cell and which sperm cell. True, many germ cells show differentiated cytoplasm, as may be determined early in the cleavage cycle by virtue of some form of granules in the cytoplasm, as in the blastomeres of the egg of *Sagitta*, *Cyclops*, etc. On the other hand, eggs of *Ascaris* show no such cytoplasmic indicators. There is, therefore, no ground for supposing that the presence of a cytoplasmic inclusion determines that a cell will become germ cell; rather, the germ-cell indicator, if such is present, may be an expression of the ground-substance itself.

Among the trematodes and cestodes, the yolk is elaborated by accessory vitelline glands and added to the egg. Here vitelline material and egg, as products of the ovary, are sharply separated. It is legitimate to consider this as no special case; rather, it is comparable to the mode by which the yolk is deposited in the egg of *Dytiscus*, of *Diopatra*, of mammals, etc. That is, during its cycle from primordial germ cell to definite egg cell, the egg elaborates reserve from food outside itself. Its specific quality of cytoplasm determines the disposition of the food so gained and the conversion of this into inclusions which themselves show some degree of specificity.

After the initiation of development, fertilization, or parthenogenesis the egg sunders itself into blastomeres. Some of these have more inclusions than others; among these latter are those whose cytoplasm appears in life as optically empty. But all—the richly laden and the optically empty—are living cells. Indeed, the tempo of life-processes is faster in those cells whose cytoplasm is less laden.

Many animal eggs exhibit in striking degree a characteristic pattern in a measure determined by the differential location of the formed bodies in the cytoplasm. These bodies, of different chemical constitution and physical attributes, occupy normally in the unfertilized egg positions independent of their specific gravity. In some eggs they are, after initiation of development, shifted to new cytoplasmic areas; this relocation cannot be ascribed to a sorting-out of bodies according to their specific gravity, for the lightest components, the oil drops, do not come to lie separately from the heaviest, the pigment, situated below the yolk spheres. Rather, the distribution, ever the same—especially a facile demonstration in eggs, as of *Chaetopterus*, or of the nereids, whose cytoplasm includes formed bodies of great variety as to form, size, and color—is without reference to the specific gravity of the inclusions. Oil and yolk are juxtaposed in these eggs with determinate cleavage; or, as in the egg of *Arbacia*, the oil drops are shuttled back and forth, now closely agglomerated, now more widely dispersed, during the cycle of mitosis, while the yolk is packed in the vegetal hemisphere of the egg, and the pigment granules are trapped at the egg surface. In other eggs, such as those of elasmobranchs, reptiles, and birds, to a disk of cytoplasm, which in the living state appears devoid of formed bodies, is pendent the mass of yolk and oil. In eggs, of cephalopods and of

teleosts a thin layer of superficial clear cytoplasm becomes, after fertilization, converted into a similar disk at the surface of the yolk mass; whereas in eggs of insects, a clear rim of surface-located cytoplasm incloses a mass of yolk and other formed bodies.

In eggs of some species of animals the granules suspended in the cytoplasmic menstruum appear to occupy positions in the unfertilized egg, when undisturbed, which are constant with respect to a line drawn from the site of polar-body extrusion to the opposite pole of the egg. Eggs exhibiting such distribution of the cytoplasmic inclusions are said to reveal polar organization, the location of these inclusions being an index of the eggs' polarity. The most striking examples of this type of organization, best seen after fertilization, are offered by eggs of the cephalopods, elasmobranchs, teleosts, reptiles, and birds; in these the cytoplasmic inclusions, such as oil and yolk, are pendent to a disk of clear cytoplasm situated below the point of polar-body extrusion, in which disk alone the initial cleavages of the egg occur. In such telolecithal eggs, so-called, one might say, if they are radially symmetrical, that the spherules and granules in the cytoplasm have a disposition indicating an organization of the egg with reference to a line drawn from site of polar-body formation to the egg's opposite pole. This, however, does not mean that in such eggs the distribution of cytoplasmic inclusions determines polarity or that polarity depends upon this distribution. The cause of polarity in these eggs is independent of the presence of these bodies. This statement is supported when we consider all animal eggs.

Properly to envisage the problems of general embryology, problems common to all animal eggs, one ought to include every egg known to us by descriptive studies and ought to appraise experimental studies on eggs of individual species with special reference to these general problems. Such study demands a most exact knowledge of normal processes and of what is peculiar and specific to a given egg. We should guard against the mistake of attempting to interpret the grand problems of embryology by descriptive, and especially experimental, study on a single species of egg—viewing, as it were, the whole field of animal embryology through the body of a single egg and obtaining thereby a distorted image of the truth.

Many eggs, both before fertilization and during the cleavage stages subsequent to fertilization or the parthenogenetic initiation of development, never exhibit any constant relation of the arrangement of oil, yolk, and other bodies to an imaginary straight line running from the point of polar-body extrusion to the pole opposite. In such eggs, other indexes are used to discern polarity. The term "homolecithal," applied to eggs in which the yolk spheres are uniformly dispersed, indicates that animal eggs exist in which the distribution of the yolk is not oriented with respect to a polar axis.

One denominates as "centrolecithal eggs" those in which the yolk appears massed around the egg center, as in a medusa or an insect egg. Such an egg, even if it is radially symmetrical, owes its polarity to some other factor than that of the distribution of yolk. The location of the yolk, taken alone, is no evidence for polarity in centrolecithal eggs.

Among flatworms are found some species of eggs to which yolk is added by vitelline glands. Here, polarity presumably is inherent in the organization of the egg. It is unlikely that the adventitious disposition of material from accessory glands determines polarity.

Further, any egg properly denominated as one having a polar axis must obviously exhibit polar symmetry. However, there exist eggs which are bilaterally symmetrical;

these cannot, just as obviously, possess polarity in the sense here discussed—i.e., in them no line can be drawn to the points on which the cytoplasmic inclusions can be related radially. Bilaterality relates to a plane and not to a line; eggs are, moreover, figures in three planes of space, they are never lines or planes.

The distribution of yolk would indicate polarity only in a radially symmetrical telolecithal egg. Since no such egg exists, the location of yolk is neither a factor nor an indicator of polarity in animal eggs. It thus follows that yolk plays only a secondary role in the organization of the egg and in embryogenesis.

This conclusion is further supported when we consider the fate of the yolk sac in the mammalian egg. A vestigial structure, it plays no role comparable to that of the yolk in a cephalopod or in an insect egg or to that of the yolk mass added to a trematode or cestode egg. In lieu of the transformation of yolk into protoplasm, the mammalian egg elaborates protoplasm from the maternal blood.

The situation is therefore this: All animal eggs obtain nutritive substances from without. In some the material metabolized as yolk is accessory to the egg; and in others, as in the mammalian, the preponderance of the source of energy for the stages of development subsequent to the initial period is derived from the blood directly. All this indicates strongly that yolk, as such, is not protoplasm, although it is the raw stuff from which protoplasm builds itself up.

Said otherwise, yolk (and oil) is food material to tide over the developing egg during the period which intervenes after the completion of the energy changes embracing the processes by which the embryo is laid down and before the advent of those depending upon the intake of food. Examples of this type are eggs with total cleavage, such as those of echinids and worms; these utilize no oil and yolk until the gut is laid down. As larval forms, they are unable normally to hydrolyze food given them before the yolk and oil in the gut cells have been broken down. The eggs of teleosts or of birds represent another class, that in which the initial plaque of clear cytoplasm, the seat of the cleavage process, is augmented by the building-up of protoplasm from the subjoined yolk area. The extreme cases, I repeat, are the eggs of some flatworms and the mammalian egg; in the former, the yolk is not a product of the ovary but of an auxiliary gland; in the latter, the egg is denuded of yolk by the formation of the yolk sac. Within these limits one may range all other eggs on the basis of the distribution of the yolk and, better, on the basis of the time during the early stages of development—after complete cleavage, homolecithal eggs; and during cleavage, telolecithal and centrolecithal eggs—when to the clear cytoplasm is added new cytoplasm elaborated from the yolk. The question is not only the spatial distribution of yolk in eggs but also its utilization early or late in the egg's development. From this emanate problems of fundamental significance for the dynamics and energetics of the embryogenetic process. This nonconstant distribution of yolk in animal eggs is a fact to be reckoned with by those who still maintain that cytoplasmic inclusions are, per se, the living units. From this point of view, the yolk of the trematode egg, being extra-ovarian, the egg, as ovarian product, would be auxiliary only; and the yolk sac would endow the mammalian egg with life.

If we envisage more completely the spatial distribution of the yolk in eggs, we note that this is not so varied as the terms "homolecithal," "telolecithal," and "centrolecithal" seem to imply. No animal egg exists in which the yolk is not completely inclosed by the clear layer of superficial cytoplasm, the ectoplasm, itself free of yolk. In all animal eggs the yolk is located in the endoplasm. The centrolecithal eggs encountered

among coelenterates and arthropods are thus no extreme configurations; rather, they represent states in which the conditions common to all eggs are strongly emphasized. That is to say, all animal eggs, except those like the trematode and the cestode egg, are centrolecithal, some more sharply than others, the superficial rim of cytoplasm being more extensive in some than in others. Even the telolecithal egg may be considered centrolecithal, for in it, best seen before the appearance of the cytoplasmic disk, a fine rim of superficial cytoplasm incloses the yolk. Only such eggs as those of the trematode and the cestode are truly alecithal. But their existence tells us that yolk, as such, is not a necessary and absolute essential for the egg cell.

The distribution of yolk thus has no fundamental significance for the egg's development, the types of distribution showing no basic differences. That yolk plays a secondary role in development is also shown by experiment.

Experimentally homolecithal eggs can be rendered telolecithal without any disturbance of the normal course of development. Of the experimental means—staleing, temperature changes, and centrifuging—the last named is best, since by it the egg may be least injured.

If, before fertilization, one centrifuges an echinid egg, such as that of *Arbacia* or that of the polychaete *Chaetopterus*, the cytoplasmic inclusions are massed according to their specific gravity; the oil drops at one pole; the yolk at the other, above pigment granules, the heaviest of the inclusions. Except for the disposition of the oil drops, such a centrifuged egg strongly resembles a telolecithal egg, such as that of a teleost, with the difference, of course, that the centrifuged egg cleaves totally, whereas the teleost egg cleaves only partly. Any egg susceptible to innocuous centrifuging shows the same picture.

Centrifuged eggs whose inclusions show changed locations cleave in all possible manners with respect to the axis of stratification: the first cleavage planes may pass along this axis or at any angle to it. The irrelevance of the plane of division to the axis of stratification is demonstrated also by the position of the polar spindle in eggs fertilizable in the stage of first maturation: in the egg of *Chaetopterus*, for example, the first maturation spindle takes any position with reference to the axis of stratification.

These findings on centrifuged eggs, whether these develop with or without cell lineage, demonstrate sufficiently that the altered disposition of the cytoplasmic inclusions in no wise obstructs the normality of development. The inclusions play a subsidiary role in the embryogenetic process. Indeed, the displacement of yolk, which in the normal egg comes to lie in the gut cells, in no wise interferes with the viability of the larva and indicates that normally yolk-filled cells, now yolk-free, possess the full complement of vital activity. In my preparations of blastulae, gastrulae, and plutei of centrifuged *Arbacia* eggs the yolk is found in mesenchyme, ectoderm, or other cells, including those of the gut. Thus, the yolk is a passive component: displaced to other cells than those of the gut, or lacking to the gut, it does not hinder or vitiate the normal processes of development. The locus of the embryogenetic forces is to be sought in the menstruum in which the inclusions lie. This is the ground-substance.

The occurrence of normal polyembryony, as in insect and armadillo eggs, is evidence of the potency of the clear cytoplasm for the unfolding of the embryogenetic process, for the embryos develop, in the former, from the superficially located clear cytoplasm and, in the latter, from the disk of clear cytoplasm. Not only is the clear cytoplasm sufficient

for the transformation of egg to early embryonic fundaments; it is, in normally polyembryonic insect eggs, capable of giving rise to hundreds of embryos.

Experimentally a normally monoembryonic egg, fragmented before fertilization, may, after fertilization of the fragments, give rise to several embryos. The fact that these egg fragments, when fertilized, develop normally no matter which inclusions they may hold indicates again that it is not the inclusions but the material in which they lie, the clear cytoplasm, which is the seat of the developmental forces. The classical work of Delage and others on merogony admits of this conclusion.

If, against these considerations, one raises the objection that in some eggs, notably those with cell lineage, such as the eggs of *Dentalium* and of *Patella*, blastomeres which were separated after first cleavage develop defectively, thus indicating a differential distribution of embryonic potencies—in some cases revealing a spatial pattern of materials at first cleavage which is similar to what would be the one or the other embryonic region in the intact egg—the very clear answer is that in such eggs the polyembryonic condition is at first cleavage already replaced by the restriction to monoembryony. So far, experiments on fragmenting eggs before fertilization and on fertilizing these fragments admit of no categorical distinction between determinate and indeterminate eggs. Fragments of an unfertilized egg of *Chaetopterus*, cleaving with definite cell lineage, develop when fertilized, as well as those of *Arbacia* or other echinids whose cleavage shows no cell lineage. From the fact, as Teitelbaum has shown, that from an egg of *Chaetopterus*, when experimentally treated during the cycle of first cleavage, twin-embryos are called forth, can be adduced a further argument as to the similarity of indeterminate and determinate eggs. This finding and a similar one on the egg of *Tubifex* constrain us to conclude that the polyembryonic capacity of animal eggs is lost sometime after fertilization; eggs differ in time after fertilization when this loss occurs. Elsewhere⁵ I have called attention to the two serious sources of error which place under grave suspicion the validity of Hörterstadius' results on the development of isolated blastomeres of echinid eggs. But, even if these results are correct, we still need to bear in mind that, so far as we know, all animal eggs capable of experimental fragmentation are, before fertilization, potentially polyembryonic. This potency resides not in the cytoplasmic inclusions but in the menstrium in which they lie, that is, in the cytoplasmic ground-substance.

Experimental results of another category put this conclusion beyond question. I refer to the developmental capacity of inclusion-free egg fragments obtained by centrifuging unfertilized eggs. Although experiments demonstrating that the potency of such clear fractions of cytoplasm (with or without the egg nucleus) is equal to that of the whole egg cytoplasm are all too few, the evidence obtained from them suffices to warrant a conclusion of wide application.

The original work on the development of fragments of nearly inclusion-free cytoplasm was done by Lillie on the egg of *Chaetopterus*. Later, Wilson confirmed and extended Lillie's observation. These workers found that the unfertilized egg, when centrifuged, is first drawn out into a thread; by further centrifuging, the thread is broken. Thus are obtained from an egg one fragment containing all the inclusions save the oil drops, and one, the clearer fragment, containing the oil drops, which Lillie mistook for the residual substance of the germinal vesicle. Lillie's work clearly showed that the hyaline frag-

⁵ E. E. Just, "A Single Theory for the Physiology of Development and Genetics," *Amer. Nat.*, Vol. 70 (1936).

ments of the egg of *Chaetopterus* cleave as do whole eggs, exhibiting the same cell lineage. Thus, for this egg at least, it is true, beyond doubt, that the hyaline substance of the cytoplasm separated from the inclusions, save oil, is capable of embryogenesis. Presumably, pieces below a minimum size would not develop into larval worms. What this limit is ought to be determined, because it would furnish an elegant starting-point for the resolution of several interesting problems in embryogenesis, as well as in the biology of the cell.

My own observations, made on echinid eggs, for the most part at Naples, indicate that the clear cytoplasmic fragments are in no wise inferior to inclusion-rich fragments in developmental capacity. To obtain the fragments, I take two lots of unfertilized eggs, one centrifuged, the other not. A sample of each lot is shaken up with bits of a broken glass cover slip. In the case of the centrifuged eggs, it is a simple matter to remove with a pipette the clear fragments, which, however, often contain the cluster of oil drops. This method, very crude though it is, is preferable to that of obtaining the fragments by centrifuging the eggs in sea water plus sugar solution, because of the toxicity of such sugar-sea-water solutions. Perfectly hyaline fragments obtained from centrifuged eggs develop as well as fragments from uncentrifuged eggs. For the egg of *Chaetopterus* I merely follow Lillie's simple method of centrifuging in sea water at that speed and for that time necessary to fragment the egg. By shaking up pieces of clear fragments with bits of a cover slip, I have obtained clear fragments free of oil drops, which are as capable of development as those containing the oil. I have also studied egg fragments of *Amphioxus* with inconclusive results, owing, doubtless, to the rapidity with which this egg loses normality after having come into sea water.

The conclusion of the foregoing is that the real cytoplasm, the living substance per se, is the hyaline menstruum in which the various formed bodies lie. It, with or without the egg nucleus, develops perfectly if fertilized. Thus we conceive of the egg cell as an organization of this cytoplasm, or ground-substance, plus one gamete nucleus. Up to now there is no single datum worthy of credence which indicates that an animal egg, devoid of one or the other germ nucleus, can develop into a larval form, although we know from the work of Vatsu and of others that enucleated egg cytoplasm can cleave.

The conception of the cytoplasmic ground-substance as the living substance par excellence has far-reaching implications.

The normal egg portrays, in one way or another, a kind of orientation of its structural components, as shown by a definiteness of sequence in time and of order of the developmental process in space which fixes the embryogenesis of each species—so definitely that many speak naïvely of the egg, remembering the course previously followed by its innumerable ancestors. This orientation resides in the ground-substance, which shifts the passive nutrient substances suspended therein to new positions. For some biologists this organization is polar. In biology the term "polarity" ought not be used, as in physics, to mean a directed attraction of visible particles; there is no evidence that the formed bodies in the cytoplasm are polarized—any more than there is evidence that in any single species of egg the embryo forms along a line running from one pole of the egg to that opposite. Such an egg as that of a trematode is, strictly speaking, devoid of yolk particles; an egg fragment free of inclusions develops as the whole egg. From this it follows that the orientation, or organization, of the cytoplasm is independent of yolk and other inclusions.

What shall we say of the fact that fractions of eggs, when fertilized, develop as the

whole egg, thereby revealing that they possess an organization identical to that of the whole egg? Does not this behavior, so closely comparable to that of a magnet when cut up into several pieces, each of which retains magnetic power, suggest polarization in the ground-substance? Perhaps. But until we know beyond question that the fragments present "north" and "south" poles along the original egg axis, we cannot here speak definitely. It is just as likely that in the fragments the "north" and "south" poles represent points on the surface of the intact egg, and that the resemblance of the egg's polarity to a magnet is only superficial and without significance. Observation and experiment could put this question beyond the range of doubt.

Or, take the problem of specificity. Doubtless, cell inclusions, such as oil, yolk, mitochondria, pigment granules, are in large measure specific, differing in different species in form and in size, and in physical and in chemical constitution, as can be shown by direct observation on both living and properly fixed eggs by their physical changes under experimental conditions and by chemical analysis. However, this specificity has its origin in the specificity of the ground-substance. Without tedious repetition of what has gone before, I need merely point out that the varying modes by which yolk is stored in eggs or energy is derived, as in mammalian eggs, from the maternal blood, indicate that eggs, except those of trematodes and cestodes, convert yolk into a structure specific to the egg. In this wise eggs build up yolk; yolk never builds up eggs.

Protoplasmic specificity is also indicated by the nucleus in those systems possessing such. Since, however, not all protoplasmic systems contain nuclei, it would not be safe to assert that specificity is the attribute of the nucleus alone. Rather, on the ground of our knowledge of the existence of nonnucleated protoplasmic systems and on the basis of what we know of the elaboration of nuclear matter in eggs during cleavage, we can, with safety, assert that the hyaline ground-substance of the cytoplasm is the locus where nuclear stuff is elaborated and where nuclear specificity has its source. To this point I return later.

By structure, as by activity under normal and experimental conditions, the ectoplasm of eggs also displays specificity. Since no protoplasmic system, cell or otherwise, exists without ectoplasm, we may assume that the ectoplasm plays an important role in specificity. This, however, derives from the fact that the ectoplasm is peripherically located, differentiated, ground-substance. Nuclear and ectoplasmic specificities such as that of the inclusions are thus derivatives.

When one considers the problem of specificity in all its aspects, one can only conclude that specificity—be it of organisms, both plant and animal; of tissues, meaning how in a named species of organism one tissue differs from another; or that form of specificity which we recognize in immunity reactions—resides finally in the fundamental organization of the individual cells which make up the organism. We recognize these kinds of specificity by relatively gross indexes; even the specificity exemplified by immune reactions, since these show themselves to us by indirect means, we may consider grossly indicated.

By external form, color, size, etc., we distinguish one species of animal from another; or by their behavior—in the case of bacteria, for example—we separate forms morphologically closely alike. But these identification characters in themselves do not fundamentally constitute specificity characters. Human fingerprints, so sharply individual, by no means permit us to say that only in the whorls and ridges of the fingers resides specificity. Here the source of the specificity is the same as that which determines that

an egg or a clear fragment thereof develops most strictly along the way that for countless generations eggs of this species have developed.

Within the field of the specificity of a named species of egg—i.e., within the cytoplasmic ground-substance—arise tissues each of which carries, below the commonly employed diagnostics for their recognition, some organization in which alone its specificity has its source. We can distinguish the human parotid gland from the submaxillary or the sublingual, and any one of these from the pancreas, either in the entirety of the gland or by microscopical examination. But in each is a fundamental ground-plan which is the true and the only source of specificity whence arise the indexes of size, form of cell, distribution and quality of the cytoplasmic inclusions, and the activity of the cellular secretions.

So with immunity reactions. The use of the blood of an individual for the purpose of determining immunity to typhoid, for example, does not at all mean that the blood is the primary source of immunity and susceptibility to the disease. Similarly, phagocytosis, even if it were the sole mechanism of defense against disease-carriers, is not limited to the white blood cells only. Why one individual is more susceptible than another to disease, drugs, etc., is a problem the solution of which is to be found not by investigation of the gross means for the recognition of immunity and susceptibility but by study of the very source whence spring these states. The specificity indicated by immunity reactions is a property of the protoplasm of individual cells or their ground-substance. What is normal ground-substance ought to be so strictly determined that we can recognize this also for the human being. Once the range of normal variations is known, we may be able to determine how the protoplasm responds when disease enters. A pressing problem in medicine is thus the most exact recognition of states of health and disease as shown by the ground-substance of the cells. The poverty of methods of microscopy employed by medical workers need not satisfy us, however much these crude methods suffice for the needs of routine diagnosis.

To repeat: the inclusion-free substance of an egg is alone the living, the real, cytoplasm. When, therefore, we speak of the "structure" of cytoplasm we should adhere to this definition, dismissing thereby the alveolar, filar, granular, or other theory of protoplasmic structure which includes the entire cell contents. To postulate a plausible and practicable theory of the structure of cytoplasm we need to ascertain what the biological structure of the ground-substance is. Lack of this knowledge hinders not only biological conceptualization but also investigations by chemists and by physicists of the chemical structure and the physical attributes of the living substance.

II

Whatever the form of the protoplasmic system—without formed nucleus, or with one or more nuclei, be it of plants or of animals—it possesses surface-located cytoplasm. No protoplasmic system exists without an external limiting surface. Thus, the ectoplasm, an extension and differentiation of the ground-substance, is a *sine qua non* of living substance.

In bacteria with or without nuclear granules, the ectoplasm is a region qualitatively different from the inner core of the organism. The classes of Protozoa are most readily distinguished by their ectoplasmic structure. Thanks to Harrison, founder of the method of tissue culture, we possess valuable data concerning the physiology of the ectoplasm,

especially in the outgrowing nerve fiber. Viguer and others have extended our knowledge of the ectoplasm in tissue cells generally. For animal eggs I have reviewed observations on the occurrence of ectoplasm as found in every animal phylum.⁶

For egg cells we may note that the ectoplasm has a structure that is basically the same, namely, a series of projections of the surface cytoplasm inclosed by a fine membrane, product of the filaments. Upon this basic structure are imposed specific structural differences, such as length and thickness of the filaments and their disposition with respect to each other. In addition there are characteristics determined by the specific structure of the ground-substance of which the ectoplasm is a differentiated extension, displaying physical properties due to its peripheral location, which places it in direct contact with the cell's environment.

But it is the activity of the ectoplasm which constitutes its most prominent feature. It is the medium of entrance of gases, water, and foodstuffs from the external medium, and the gateway for the exit of gases, water, excretion, and secretion from the cell. Its role in such processes as fertilization, parthenogenesis, cell division, and differentiation is likewise clear. Thus, its role is not merely a passive one, owing to its peripheral location; it engages actively in the physiology of the cell. Moreover, it is the most readily prehensible cell region, revealing changes in normal processes easily susceptible to reversible experimental conditions and first affected by noxious treatment. Study of ectoplasmic activity is study of the most manifest, most easily tangible activity of differentiated ground-substance.

Whereas every protoplasmic system, cell or other, is a defined structure with a limiting boundary within whose confines the size of the system is set as a specific character, protoplasmic systems may or may not contain one or more discrete nuclei, as has been said above. Thus, the discrete nucleus is not a *sine qua non* of living substance. That the nucleus has specific structure is shown by the number, form, and size of the chromosomes, as well as by the form and size of the nucleus as a whole and by the structure of the mitotic spindle, when this is present. With the nucleic acid common to all plants and with that common to animals is conjugated a simple protein whose ensemble of amino acids makes the nucleoprotein a specific chemical compound. Because of chemical structure, the nucleus of every cell reveals physical attributes; presumably, therefore, these differ with different species.

In the developing egg there can be no doubt that nuclei are products of the ground-substance of the cytoplasm. The evidence supporting this proposition follows:

For those eggs upon which observations have been made, the ratio of volume of nuclear substance to cytoplasm steadily changes during cleavage, the total volume of nuclei increasing, the amount of cytoplasm decreasing. This statement holds for eggs of echinids, of mollusks, of nereids, and of *Amphioxus*. The measurements of nuclear increase should be begun at the time of first cleavage, because this is the first stage in which eggs are alike with respect to nuclear makeup, whether they are fertilized in the germinal-vesicle stage, during first maturation, during second maturation, or after maturation. The measurements ought be continued throughout the stages during which the eggs derive no food material from the environment; since eggs vary with respect to the time when yolk is transformed into hyaline ground-substance, and this depends upon the distribution of the yolk within the egg, the originally present hyaline ground-substance serves as a direct source for the nuclear increase for a period which differs with

⁶ E. E. Just, *The Biology of the Cell Surface* (Philadelphia: Blakiston, 1939).

different eggs. These measurements end at the stage in which the developing egg derives food from its environment, be this nonliving or, as in the case of the mammalian egg, living. Obviously, with the ingestion and assimilation of foodstuffs, the synthesis of nucleoprotein, as that of other compounds, cannot be directly attributed to the original cell contents.

Eggs of echinids, of nereids, and of *Amphioxus* represent the simplest cases of nuclear growth at the expense of the ground-substance. Beginning with a ratio of nucleus to total extra-nuclear substance of about 1 to 1,000, the ratio steadily falls to around 1 to 3. As far as one can judge, the ratio is similar in the egg of *Crepidula*; at any rate, I have found that in the egg of *Aplysia* the increase of total nuclear content is of the same order as in these simplest cases. The difference between these and the molluscan eggs (of *Crepidula* and of *Aplysia*) lies in the fact that, in the latter, eggs with unequal cleavage, yolk is steadily being transformed into ground-substance during cleavage, whereas, in the former, the food reserves are used only later, i.e., after gastrulation. Eggs of cephalopods, arthropods, fishes, reptiles, and birds constitute the category of eggs with partial cleavage; in them the early utilization of yolk by the ground-substance is more marked. Finally, there are mammalian eggs, on the one side, and trematode and cestode eggs, on the other; in the first named, yolk is accumulated in the yolk sac, while in the second, cleavage is apart from the yolk whose origin is extrinsic to the egg.

Adding to the foregoing the fact that fragments of clear egg cytoplasm devoid of yolk synthesize nuclei, we reach the conclusion that nuclei are derivatives of the cytoplasmic ground-substance. Hence, nuclei are derivatives, differentiations, of their inclosing cytoplasmic ground-substance. This applies to all animal eggs.

This discussion of ectoplasm and of nucleus, derivatives of the cytoplasmic ground-substance, allows us now to define protoplasm: Living substance or protoplasm is the hyaline menstruum which pervades the entirety of the protoplasmic system and in which formed bodies are suspended; at its surface it shows always differentiated hyaline substance; often it contains one or more nuclei, differentiations of the hyaline menstruum.

III

What is meant by the "chemistry of protoplasm" depends, first of all, upon what is meant by "protoplasm." That which is to be subjected to chemical analysis ought not be needlessly burdened by substances additional to the material to be studied. No true chemistry of protoplasm, as defined above, has yet been made. Indeed, chemical investigations of protoplasm, even when this is considered as embracing the entire content of the protoplasmic system, cell or otherwise, have been embarrassed by the fact that the material analyzed has not always been free of contamination. This is especially true of tissue analysis, since with tissues most often are associated blood, body fluids, excretions, etc. The chemical analysis of single cells, demanding large numbers, similarly often includes the circumambient fluid in addition to the inert cell membranes no longer part of the true cells. A chemical analysis of protoplasm in our meaning is a chemical analysis of the ground-substance.

All that has been done chemically on protoplasmic systems *en gros* needs to be done on the ground-substance alone. Until such studies have been made, we have no chemistry of protoplasm but only a chemistry of the mixture within the boundaries of the system (and often including that of these boundaries). To obtain a conception of the chemistry of the ground-substance of cells from studies so far made, one may substract what is

known of the chemistry of the cytoplasmic inclusions and of that of the nuclei from what is known of the chemistry of the entire cell contents. It would be far better, however, to analyze directly the cytoplasm itself.

In view of the elegant researches recently made, and now under way, on the chemistry of proteins, thanks to the technique of X-ray spectroscopy and of ultracentrifuging, the reader may think my position too extreme in discounting the value of our present-day knowledge of the chemistry of protoplasm. Nevertheless, I maintain this position, for I do not see, despite the great advance in protein chemistry, that we have progressed very far beyond the stage reached when Delage wrote forty-five years ago. As a matter of fact, the work on protein chemistry serves pointedly to exemplify what, to me, is the greatest shortcoming of the chemistry of protoplasm, as usually defined.

Proteins in test tubes, brutalized by the procedures necessary for their analysis, are not the same as those in the living protoplasmic system. This being so, the beautiful researches on proteins by means of spectroscopy and ultracentrifuging inform us only of the structure of changed proteins. According to some workers, it makes no difference whether one works with denatured or nondenatured proteins. But, however slightly the protein may be changed, it is changed; and therefore it is something other than that in the intact protoplasmic system. If we could reconcile the various theories proffered to-day for the spatial structure of proteins—theories some of which are highly speculative—we should still need to reckon with the fact that proteins extracted from protoplasm are different from proteins in the viable protoplasmic system. Gelatin, the white of the hen's egg, and even the globulins and albumens of blood are extractives only: having been excreted, they are no longer part of the living protoplasm.

It is no escape from this situation to speak of "living" protein in this connection. Proteins exist as molecules; we have no evidence for the existence of life-molecules. If there are such, they are probably not protein only, since we have no knowledge of any life-thing composed of protein alone. Protoplasm, as matter in the living state, is a *mélange* in which react not only proteins but also carbohydrates, lipins, electrolytes, and, most abundantly, water. Therefore, not only should we avoid confusing protoplasmic extractives with protoplasm itself; we should also be wary of taking one protoplasmic component for the whole protoplasm. For any and all of the partners in the structure of protoplasm we need to recognize the differences between these as single entities when extracted for chemical analysis and these same in that close juxtaposition in which alone resides the living state. The physical and chemical properties of the single components in living protoplasm are not, of necessity, the same as the properties of these components in a mere physical mixture. The chemical activity of the components, when assembled as living protoplasm, probably differs even more from the activity of these components when taken singly than the activity of a molecule of lipin, of protein, or of polysaccharide differs from the activity of the constituent fatty acid and glycerol, the amino acid, or the simple sugar, respectively, when taken alone. Even the electrolytes, when separated, probably do not act as they do when in conjunction with each other and with other constituents in the protoplasmic system. Who knows whether water outside a cell has precisely the same properties as water within it? Here, again, one does not avoid the problem by speaking of "living" water, a term which means nothing as long as we do not know what life is. Until we possess this knowledge, it is idle to seek an answer to this perennial riddle by hypothecating to single protoplasmic constituents, removed from their union, the life-principle which, so far as we

know, resides only in the whole, intact protoplasmic system. What is said of the proteins isolated from the intact cell, or other form of protoplasm, may be said of any component of it removed from its normal combination. Thus, here pure chemical analysis defeats itself by virtue of its purity: living protoplasm is not, in this sense, "pure."

However, to close up the gap in our knowledge of the chemistry of protoplasm, we should not merely carry over to the study of the ground-substance methods utilized heretofore on the whole protoplasmic contents. We need, rather, to adopt methods applicable to the living protoplasm itself and devised for it, however difficult it may be to place ourselves in a new way. Indications are not wanting to show that it is possible to study the chemistry of ground-substance directly in the living condition, although at present the methods are, of necessity, crude and only qualitative.

A word about enzymes may not be amiss here. Work such as that by Holter on the localization of enzymes within cells shows that the ground-substance alone contains them. Extension of this work will doubtless give clues of value concerning the chemical composition of living protoplasm.

The enzymes best known chemically are either simple proteins, such as pepsin and trypsin, or conjugated proteins, as, for example, the respiratory ferment (oxidative catalyst). If it should prove to be true that all enzymes are proteins, we can say that the intraprotoplasmic chemical reactions characteristic of living substance probably are due to some peculiar protein bodies. Adding that bacteriophage is perhaps a conjugated nucleoprotein and that an ultrafilterable virus isolated by methods used for the purification of pepsin and trypsin appears to be an enzyme, we conclude that certain proteins play a definite role in intraprotoplasmic reactions. These reactions, however, involve other compounds than proteins, since not all the chemically known enzymes are proteolytic; they break down (and build up) other constituents—carbohydrates, fats, and still simpler compounds—and hasten oxidation. Thus, urease is not a proteolytic enzyme. Undoubtedly many intraprotoplasmic reactions are autocatalytic; as such, they demand precursor reactions, since they do not start of themselves. The study of autocatalysis in living systems is therefore strongly indicated. One such lies close at hand, the synthesis of nucleoprotein, as revealed by the increase of nuclei during the early stages of an egg's development. In the cytoplasmic ground-substance reside the precursors of the nucleoprotein which constitutes the nuclei.

Enzymes act singly or with coenzymes to break down or to build up compounds. Some, such as pepsin, trypsin, etc., are extracellular, acting after having been excreted by the protoplasm; others, the intracellular, offer more difficulty for study. Until more is known of the latter, comparison of their activity with that of extracellular ones is hazardous. Much of the work on intracellular enzymes I consider doubtful, for the reason that it is done on the enzymes after they have been isolated, the protoplasm having been destroyed. Such work on intracellular enzymes concerns itself with the presence of coenzymes, the speed of enzyme reaction, and the quantity of enzymes present. With respect to the first, the methods of isolation may induce formations interpreted as coenzymes. Further, the speed of reaction within a cell and in a test tube may be different. Up until now, our ignorance of the underlying physical conditions which contribute to the velocity of protoplasmic reactions is too great to warrant an assumption as to the identity of speed of reaction in living protoplasm and in test tubes. Finally, the quantity of enzyme present does not always give the rate of reaction or indicate that reaction takes place.

It cannot be overemphasized that what we need in the chemical study of the ground-substance is chemical analysis not only of the dead stuff but also of the living. The latter is difficult but not impossible. Such chemical study should include, first, the mapping-out of the pattern of the ground-substance specific for each protoplasmic system. Animal eggs furnish excellent objects for this type of investigation.

Results of my own, as yet unpublished in detail, belong here. By means of staining and of precipitation reactions I have been able to relate the intimate structure of the ground-substance to the specific organization of a given egg, as well as to the changes in a named species of egg that occur during the mitotic cycle. Spectroscopic investigation of the ground-substance is also possible, as I have found.

By means of studying the dispersion, appearance, and disappearance of water drops in cells I have been able to obtain a clue concerning some enzyme reactions in living, viable egg cells. This, an indirect method to be sure, is excellent for investigating hydrolysis and synthesis occurring within the cytoplasm. With it I have been able to study, for example, the hydrolysis and synthesis of lipin in living endoderm cells, without disturbing in any wise the normal processes of development. Further, the appearance and disappearance of water drops in perfectly normal cells furnishes a reliable index for the onset and direction of rhythmical protoplasmic behavior.

The progressive synthesis of the nucleoprotein, as this is shown in eggs during cleavage by the progressive increase of nuclear material, also demands our attention.

The studies made with the Feulgen reaction and those made by Caspersson are aids in the investigation of nuclear material. The methods of Caspersson and similar ones could be so extended that they would throw light on those reactions in the ground-substance antecedent to the synthesis of nucleoprotein. These, in combination with the use of the old Altmann method, as revived by Bensley and Gersh, promise much. No problem in the whole field of cellular biology carries more weight than this of the progressive building-up of nuclear matter out of ground-substance. A parallel investigation ought to be made on the growth and reproduction of noncellular protoplasts.

What has been said above concerning the biological (morphological and physiological) structure of protoplasm, as well as concerning its chemical makeup, applies equally to its physical structure. Physical studies on an entire protoplasmic system, including effete substances, food material, etc.; such studies as those on permeability; or others on single properties of single regions of the cell may have value. But there is an urgent need for the study of the physical properties of protoplasm *per se*, of the living ground-substance. Here, also, study of the rhythmical appearance and disappearance of water drops in normal cells would be of incalculable help. The too great neglect of investigation of the behavior of water, quantitatively the most abundant compound in living substance, amounts to a reproach. As long as we remain in ignorance concerning the distribution of water in the normal protoplasmic system, our theories of the physics of the living state rest, of necessity, on an incomplete foundation.

Moreover, the sooner we dismiss the theory of surface tension in biology, together with the theories of permeability, the earlier we shall approach a substantial knowledge of the physical structure of protoplasm. Surface tension, it seems, is a chemical phenomenon. At any rate, it applies to molecules. From this point of view alone, it would seem to be as dangerous to apply its use to protoplasmic systems as to electrons: the former are too complex, the latter not complex enough. One only begs the question when one speaks of "tension at the surface." Theories both of permeability and of surface-

tension in biology ignore the ectoplasmic structure of the protoplasmic system; overlooking the livingness of living things, the authors of these theories accumulate evidence from elegant experiments on nonliving membranes and inert powders spread upon water.

For biological, as well as for chemical and physical, investigations, the first question to be answered is: What is normal living protoplasm? The failure to recognize this normality may be designated as the chief characteristic of many of the current researches in chemical and physical biology. The orthodox biologist also is often guilty of being ignorant of the normal process in the system under investigation. Because of the fact that experimentalists frequently do not appreciate normal processes, their experiments, especially those that debase the normal state, often yield results of little significance for the object of the work, an explanation of normal phenomena. These processes and their range of normal variation must be fully recognized before an attempt can be made by methods of experimental pathology to interpret them. A serious responsibility rests upon the descriptive biologist to establish, beyond question, criteria of normality, the range of normal processes, and the extent of their normal variability. A physics and a chemistry of living protoplasm worthy of being so called depend upon this descriptive biology.

IV

The problems of general biology named at the outset hang upon the problem of the biological, chemical, and physical structure of protoplasm. Concerning the origin of life, the maintenance of its specific form, and its specific reduplication, we may speculate—have speculated—without having been able to place these questions in the category of those fully answered. Modern theories proffered to explain these problems of general biology ignore, if they do not discount, the basic problem of the structure of protoplasm.

These theories rest, in turn, upon one or the other of two conceptions which have dominated biological thinking and provoked various expressions of allegiance on the part of their respective adherents. I refer to the mechanistic (nowadays often denominated the physicochemical) and the vitalistic schools. In my judgment, the fight between mechanism and vitalism is in large measure a war of words, as I have elsewhere pointed out. But, if for the moment we take these terms as truly representing a joined issue, we discover yet another fallacy, which amounts to a defection in the camp whose aegis is mechanism.

In attempts at mechanistic interpretations the conception is only allegedly mechanistic, and in reality is as vitalistic as any statement of purest vitalism.

With the concept of evolution the case is clear. Evolution is a concept pure and simple, and not a demonstrated scientific truth, however much the evidence in its favor is accepted. Mechanistic explanations fail here. Such terms as "survival of the fittest," "struggle for existence," "natural selection," and the like owe their power as much to their poetical connotation as to their biological basis. Biology, we must recognize, is a welter of such figurative expressions and of physical and chemical borrowings that obtrude themselves irritatingly whenever we attempt to precisise the meanings of words employed in the science—indeed, their too abundant use often tends to place the science outside the realm of other natural sciences, rendering difficult exactitude of expression. To be sure, chemistry uses terms such as "chemical affinity," and physics such as "attraction," for example; but usage in the physical sciences has sharpened the meaning of terms, whereas in biology comparable ones carry varying imports.

Take, as another example, the concept of the axial, polar, physiological, metabolic gradient, or of physiological dominance of fields—call it what you will. These terms are merely pretensions. They seem to be physical but are only the mechanistic biologists' euphemisms for "vital force," "entelechy," "*élan vital*," etc., inasmuch as they substitute for expressions avowedly vitalistic a new term which differs only in so far as it means that life-processes are organized along an axis instead of being not so limited by the upholders of the theories of entelechy and the like.

Or, consider the concept of the gene. Many a sufferer of typhoid fever or of another infectious bacterial disease knows, to his discomfort, that bacteria hand on their heritage. Most bacteria possess no chromosomes. The gene theory does not hold for such organisms. Indeed, the theory may or may not hold for organisms containing nuclei and chromosomes. But, even if it could be proved to hold for every single living thing, plant and animal, it is far from being the physical concept claimed for it by its founder, i.e., that it is a concept built upon quantitative experience as elegant as the beautiful work on the electron. Its mathematical basis is far removed from the exquisite fineness of modern physical measurements. The gene theory, however much it pretends to be mechanistic, is only crudely vitalistic: it gives us a set of demons, the genes, capable of extricating the theorists from every difficult situation. For one entelechy it sets up many.

These alleged "mechanistic" interpretations have failed. They tell us, though covertly, that to formulate life we need to invoke terms, as "entelechy," "vital principle," "*élan vital*." The mechanists should, therefore, abandon synonymous expressions no less vital than these, because they are garbled physical concepts. There remains the way of investigating life from a *nonmechanistic* point of view, which is, however, in no sense vitalistic.

Life is not a metaphysical vagary but a phenomenon which we do not yet comprehend. The practicable point of view which, it seems to me, is most productive is that which concentrates on the visible manifestations of livingness, the tangible expressions, the phenomena. Once the phenomena are apprehended, the right conception will emerge. Certainly, up to now, our conceptions leave much to be desired.

Not less inept than these opposing mechanistic and vitalistic conceptions are the theories underlying the three above-named problems of general biology.

Certainly, nowadays no one holds to the outmoded view that study of fertilization and of experimental parthenogenesis can aid us directly in discovering how life arose. This view never would have prevailed but for the ardent faith of blind worshipers who saw in the early work on experimental parthenogenesis the creation of life. It may be that life *was* created—if so, then we be futile laborers who seek to unravel the mystery of its origin and evolution. But, if life arose and evolved, then the understanding of its origin and evolution again wait upon further knowledge of protoplasmic structure. What arose and what has evolved are the insisting and persisting questions. They concern the living substance *par excellence*, i.e., the ground-substance.

A point less disputed perhaps, but none the less important, is that of the ordered maintenance of life-forms persisting through generations. What is maintained—what persists? Here, again, the fundamental question is that of protoplasmic structure. As in every single domain of cytological investigation known to me, we see here darkly through a glass clouded by the haze of bad definition. Growth is often confounded with differentiation and—in the case of cells and especially of egg cells—with cell division. The self-regulation, self-maintenance, and self-perpetuation of the protoplasmic system

constitute phenomena of paramount importance. With heredity and evolution they are inextricably bound: they, too, have been inherited, have evolved.

To whatever extent we accept the data of geneticists, we are loathe to regard, as does the founder of the gene theory, the problem of heredity as solved. On the contrary. Until we know much more concerning the biological, chemical, and physical structure of protoplasm, the problem of heredity remains open. A theory of heredity must apply to all animals and all plants, whether these are composed of nucleated protoplasm or not. For beings made up of cells, we must know, beyond question, the origin and synthesis out of the ground-substance of the genes constituting the chromosomes in nuclei. Moreover, we must know how, upon the thread of the nucleic acid common to plants and that common to animals, the specific gene entities are imposed—unless the genes are nucleic acid. What knowledge we possess up to now only allows us to consider the chromosomes as indicators of deeper-seated processes.

In the preceding pages I have endeavored to show that the pressing problem in general biology is a clear recognition of what in any protoplasmic system, cell or otherwise, is the living substance. In default of this recognition, the questions of how life begins, how it is continued, and how transmitted will remain unanswered. Thus, knowledge of protoplasmic structure is basic for these, as yet unsolved, problems of general biology. To obtain a conception of this structure demands a concerted attack by biologists, chemists, and physicists who appreciate the limitations of the life-state, the range of normal life-processes. Said briefly, we need a biology that recognizes its field and the problems peculiar to it and which treats these according to its domain and what that domain embraces.

PHYSIOLOGY OF DEVELOPMENT OF THE FEATHER. III GROWTH OF THE MESODERMAL CONSTITUENTS AND BLOOD CIRCULATION IN THE PULP¹

(Nine text figures; four plates)

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I. INTRODUCTION

THE following investigation started out from the fact that in studies of the development of the feather attention has been directed almost exclusively to the ectodermal constituents; this was also the case in our own previous studies (Lillie and Juhn, 1932, 1938). The problems of the growth and functioning of the mesodermal constituents, pulp and papilla, of the feather germ have thus been relatively neglected.

All growth of the feather proper is dependent on the blood supply of the pulp, which bears the entire burden of nutrition; all hormonal influences affecting the form or pattern of the feather are transmitted by the blood; other chemical variations in the blood stream and variations of blood pressure must also play their part in the development of the feather.

Moreover, there does not exist anywhere in the animal kingdom, so far as I know, such extensive vascular protrusions from the surface of the body as in the pulps of

¹ The expenses of this investigation were supported in part by a grant from the Rockefeller Foundation to the University of Chicago.

growing feathers, which reach a length of at least 6 cm. in wing and tail feathers of the fowl, and probably even greater lengths in some larger birds. What, then, are the special vascular arrangements that control all this conspicuous functioning?

Such questions, with their obvious reference to our preceding investigations, furnished the starting-point and motive for the study that follows. They soon led into inseparable problems of growth.

The White Leghorn fowl has been used, for the most part, in this study, because the absence of chromatophores and pigment is a great advantage in studying the circulation; but Brown Leghorns have also been used to some extent. The adult was used generally, but occasionally the juvenile plumage was used.

The injections, the measurements, and the technical operations were performed by my research assistant, Mr. Hsi Wang, under general direction. To his skill and devotion to the work the writer is deeply indebted. The microscopic sections were made by Miss Margaret Davidson, of the General Biological Supply House. Reference is made to Figures 18 and 19 for general topography.

II. THE PULP AND THE PAPILLA (GENERAL)

The pulp is a loose mesenchymal reticulum, the interstices of which are occupied mainly by a homogeneous, gelatinous substance that gives the entire pulp a firm, tough, and elastic consistency. A colorless serum also exudes from injured surfaces of the pulp. The rich, highly complex vascular system of the pulp is described in section IV.

The pulp exerts considerable pressure on the ectodermal cylinder of the feather germ and thus keeps it in a state of tension which is responsible, among other things, for the smooth, even surface of the sheath. If a plucked regenerating feather is kept in the open air, the sheath soon wrinkles, owing to loss of water from the pulp; but the sheath remains smooth indefinitely in a moist chamber. The sheath normally persists several millimeters above the apex of the pulp. Turgor of the pulp² can readily be demonstrated by removing the sheath of a regenerating breast feather down to the mouth of the follicle with the feather *in situ* in the living bird. The exposed barbs are then unable to resist the internal pressure of the pulp, which expands and forms a swelling, tapering in an apical direction, just above the mouth of the follicle. This happens in a very few minutes. If 2-4 mm. of the sheath is left above the mouth of the follicle, a similar, but smaller, swelling develops above the sheath. The apical third of the pulp does not swell so readily, owing to increasing thickness of the pulp membrane (p. 153).

No evidences of cell division are found anywhere in the pulp itself. The cellular richness of the pulp is greatest next to the papilla³ (Pl. II, Fig. 1), and rapidly diminishes, with corresponding increase of intercellular substance, in an apical direction, and then remains practically uniform to the apex (cf. Davies, 1889, and Lillie and Juhn, 1932). The pulp, in fact, like the feather proper, grows from its base; and there can be no doubt that the cells are derived from the papilla.

The pulp arises from the papilla at the onset of regeneration of the feather and is

² The problem of turgor is discussed later; see pp. 172-73.

³ The term "papilla" is used in this paper exclusively for the persistent dermal structure underlying the pulp and filling the opening of the umbilicus. Davies (1889, p. 572 n.) used the term as synonymous with "feather germ," i.e., as including both epidermal and dermal constituents. He used the term "pulp" to include also the papilla in our sense.

finally completely resorbed when the feather is fully formed. If the growing feather is plucked, the pulp comes away, but the papilla remains.

In the papilla, apical, basal, and peripheral surfaces may be distinguished. Bundles of interwoven fibers give it a tough consistency, especially in its basal half. It is constricted about midway between apical and basal surfaces by the lip of the umbilicus (collar) (Pl. II, Fig. 1), so as to have an hourglass form.

The basal half is cleanly delimited from the derma over its hemispherical boundary, and a strong bundle of fibers is prolonged around the periphery of the base into the wall of the follicle. It is thus firmly anchored. The apical surface forms a sharper dome, the center of which may be raised around the axial artery. The periphery of the apical half sends a prolongation along the inner surface of the collar.

The apical surface of the papilla, in contrast to the basal surface, is not sharply delimited. On the contrary, it is frayed out into strands of cells that merge with the pulp. This is the region of greatest cellular richness of the pulp referred to above. This contribution to the pulp from the papilla involves not only the arched surface of the apex but also the periphery which extends along the collar, as shown in Plate II, Figure 1.

From the time of its formation the pulp is most intimately associated with the surface of the collar, though separated by the basement membrane from the epithelial cells of the collar. In the region of the ramogenous zone the basement membrane thins out and may even seem to disappear. This is a very narrow circular zone interrupted dorsally by the shaft in which the organization and cellular growth of the ridges occurs, and along which the tangential movement of the bases of the ridges is directed toward the shaft (Lillie and Juhn, 1938).

In addition to its functions with reference to the pulp, the papilla has certain morphogenetic functions. This is to be inferred by its persistence as the locus of development, replacement, and regeneration of feathers from the time of its origin in the embryo throughout life; but it is not known what morphogenetic relations actually exist between the papilla and the ectodermal component of the feather germ, and this subject is not considered in the present paper.

III. GROWTH OF THE PULP DURING REGENERATION

I. GROWTH IN LENGTH

The growth in length of the entire feather from the thirteenth day of regeneration until completion was measured at 2-day intervals for the anterior part of the breast, for the posterior part, and for saddle feathers. The length of the pulp was also ascertained for each measurement, and the percentage of the pulp length to the total length was then calculated.

The measurements for the breast feathers were made on a White Leghorn cock, which possesses the advantage that the vascular pulp can be seen *in vivo* and the same feathers and their pulps can be measured during the entire period of regeneration. The measurements given in Tables 1 and 2 are the averages of the measurements of three adjacent regenerating feathers.⁴ The data in Table 2 are shown in the form of curves (Fig. 2).

⁴ The measurements of length of the living feathers are, of course, taken from the mouth of the follicle. In order to obtain the length within the follicle, which is added, a neighboring regenerating feather of the same length as the average of the three measured *in vivo* was plucked and the length within the follicle ascertained. This is a quite constant quantity. The plucked feather was also used for determination of weight of constituents (see p. 166).

In the case of the saddle feathers, measurements were taken on Brown Leghorn capons in which the vascular pulp cannot be seen *in vivo*. This necessitated the plucking of a large area and the sacrifice of five regenerating feathers actually plucked for each

TABLE 1
WHITE LEGHORN COCK: ANTERIOR BREAST FEATHER*

Age (Days)	Total Length (Mm.)	Length of Pulp (Mm.)†	Percentage of Pulp Length to Total Length	Age (Days)	Total Length (Mm.)	Length of Pulp (Mm.)†	Percentage of Pulp Length to Total Length
13.....	16.0	11.7	73.1	34.....	58.7	12.7	21.6
14.....	17.2	12.0	69.8	36.....	63.0	13.0	20.6
16.....	21.7	12.7	58.5	38.....	66.0	10.8	16.4
18.....	25.3	12.0	47.4	40.....	70.0	10.7	15.3
20.....	29.7	11.7	39.4	42.....	73.0	10.3	14.1
22.....	34.0	11.3	33.2	44.....	75.0	8.5	11.3
24.....	37.0	11.5	31.1	46.....	76.0	7.0	9.2
26.....	43.0	12.0	27.9	48.....	77.3	5.0	6.5
28.....	48.0	12.0	25.0	50.....	79.0	3.0	3.8
30.....	50.7	12.5	24.7	52.....	79.0	2.0	2.5
32.....	55.3	12.8	23.1	54.....	79.0	0.0	0.0

* Length of pulp in relation to total length of regenerating feather, measured *in vivo*. On account of absence of pigment, the pulp can be seen *in situ*. The measurements are the average of three figures. The lengths (measured from the skin) are corrected by adding the depth of the follicle ascertained by plucking control feathers.

† The sum of all measurements of length of pulp, amounting to 215.2 mm., is referred to on p. 166.

TABLE 2
WHITE LEGHORN COCK: POSTERIOR BREAST FEATHER*

Age (Days)	Total Length (Mm.)	Length of Pulp (Mm.)†	Percentage of Pulp Length to Total Length	Age (Days)	Total Length (Mm.)	Length of Pulp (Mm.)	Percentage of Pulp Length to Total Length
13.....	18.5	12.7	68.6	38.....	81.0	13.0	16.0
14.....	21.0	13.5	64.3	40.....	85.0	12.2	14.4
16.....	25.7	14.5	56.4	42.....	88.7	12.0	13.5
18.....	31.0	15.0	48.4	44.....	92.7	12.0	13.0
20.....	35.7	15.2	42.6	46.....	96.7	12.0	12.4
22.....	41.0	15.2	37.0	48.....	100.3	11.0	11.0
24.....	46.0	15.0	32.6	50.....	103.0	9.0	8.7
26.....	51.2	14.5	28.3	52.....	105.0	6.5	6.2
28.....	56.3	14.0	24.9	54.....	106.0	4.0	3.8
30.....	61.2	14.0	22.9	56.....	107.0	3.0	2.8
32.....	66.0	14.0	21.2	58.....	108.0	2.0	1.9
34.....	71.0	13.7	19.3	60.....	108.5	1.0	1.0
36.....	76.0	13.5	17.8	62.....	108.5	0.0	0.0

* Length of pulp in relation to total length of regenerating feather, measured *in vivo*. On account of absence of pigment, the pulp can be seen *in situ*. The measurements are the average of three figures. The lengths (measured from the skin) are corrected by adding the depth of the follicle ascertained by plucking control feathers.

entry, and dissected so as to show the length of the pulp. This has the disadvantage of introducing irregularities due to different locations in the same tract. The results of measurements on two birds, as given in Tables 3 and 4 and in the curves (Fig. 3) based

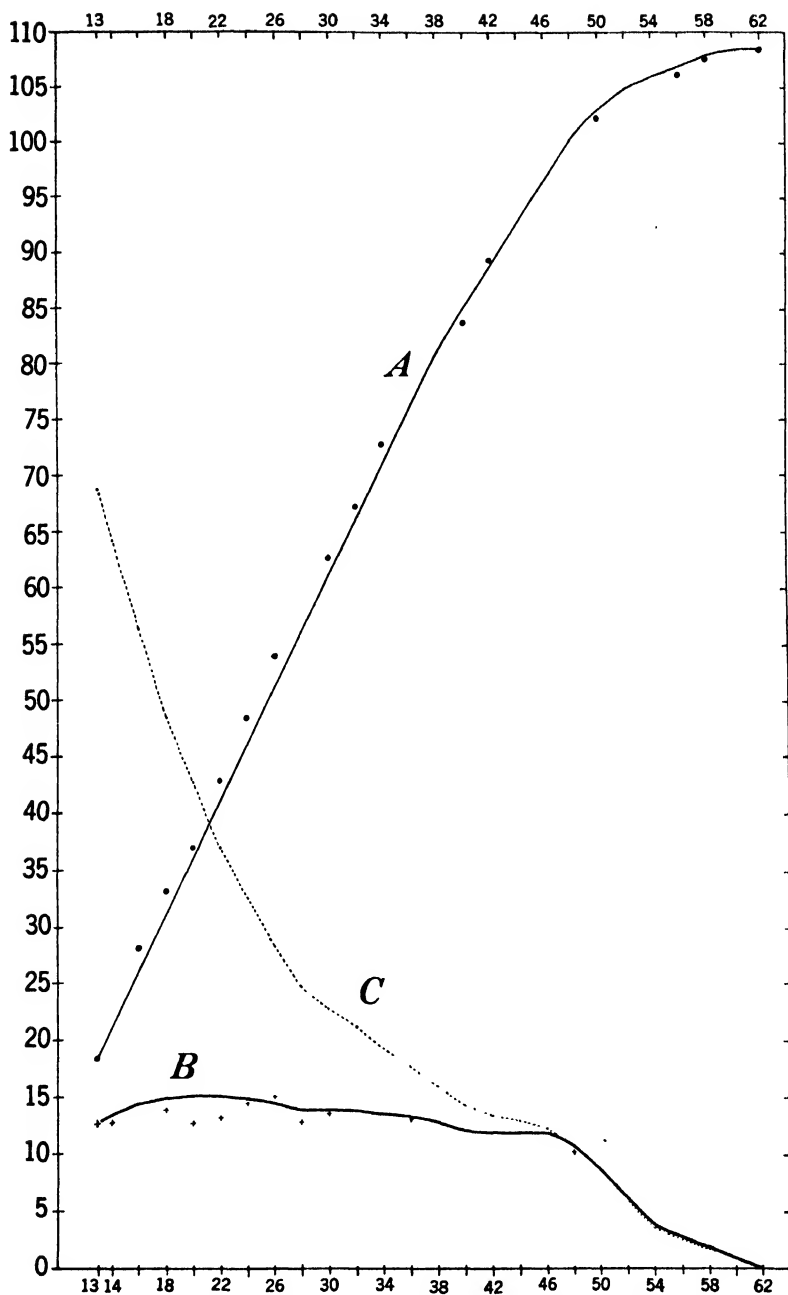


FIG. 2.—Curves of growth, feather and pulp, posterior breast feather, White Leghorn cock. Data from Table 2. *A* = feather; *B* = pulp; *C* = percentage of length of pulp to length of entire feather. Abscissae = days of regeneration; ordinates = length in millimeters for curves *A* and *B*, percentages for curve *C*.

TABLE 3
BROWN LEGHORN CAPON 171: SADDLE FEATHER*

Age (Days)	Total Length (Mm.)	Length of Pulp (Mm.)†	Percentage of Pulp Length to Total Length	Age (Days)	Total Length (Mm.)	Length of Pulp (Mm.)†	Percentage of Pulp Length to Total Length
14.....	12.5	11.5	92.0	51.....	79.3	13.0	16.4
16.....	17.0	13.7	80.6	53.....	82.7	12.5	15.1
18.....	19.7	14.8	75.1	55.....	86.5	12.3	14.2
20.....	23.4	15.2	64.9	57.....	90.0	12.2	13.6
22.....	27.0	15.3	56.7	59.....	93.0	12.1	13.0
24.....	30.5	14.8	48.5	61.....	96.2	12.0	12.3
26.....	34.3	14.2	41.4	63.....	99.5	12.0	12.0
28.....	38.0	13.4	35.3	65.....	102.5	12.0	11.7
30.....	41.5	13.2	31.8	67.....	105.5	12.2	11.6
32.....	45.0	13.0	29.0	69.....	108.7	12.3	11.3
33.....	47.0	12.8	27.3	71.....	112.0	12.5	11.2
35.....	50.5	12.8	25.3	73.....	115.0	12.7	11.0
37.....	54.0	12.9	23.9	75.....	118.0	12.0	10.2
39.....	57.5	13.0	22.6	77.....	120.2	10.7	8.9
41.....	61.0	13.1	21.5	79.....	122.5	8.7	7.1
43.....	65.0	13.4	20.6	81.....	125.0	6.2	5.0
45.....	68.5	13.7	20.0	83.....	127.0	3.0	2.4
47.....	72.0	13.6	18.9	85.....	129.0	0.0	0.0
49.....	75.5	13.5	17.9				

* Length of pulp in relation to total length. The measurements are the average of 5 plucked feathers for each entry.

† The sum of all measurements of length of pulp, amounting to 443.3 mm., is referred to on p. 165.

TABLE 4
BROWN LEGHORN CAPON 148: SADDLE FEATHER*

Age (Days)	Total Length (Mm.)	Length of Pulp (Mm.)†	Percentage of Pulp Length to Total Length	Age (Days)	Total Length (Mm.)	Length of Pulp (Mm.)†	Percentage of Pulp Length to Total Length
13.....	11.5	10.5	91.3	49.....	78.0	13.0	16.7
15.....	15.5	14.0	90.3	51.....	81.5	12.5	15.3
17.....	19.0	15.5	81.6	53.....	85.5	12.2	14.3
19.....	20.5	15.2	74.1	55.....	89.0	11.8	13.3
21.....	26.5	14.8	55.8	57.....	93.0	11.5	12.4
23.....	30.5	14.4	47.2	59.....	96.5	11.2	11.6
25.....	34.0	14.0	41.2	61.....	97.5	11.3	11.6
27.....	37.5	13.8	36.8	63.....	100.5	11.5	11.4
29.....	42.5	13.5	31.8	65.....	103.0	12.0	11.7
31.....	45.0	13.2	29.3	67.....	105.0	12.5	11.9
33.....	48.5	13.2	27.2	69.....	107.5	13.0	12.1
35.....	52.5	13.5	25.7	71.....	109.5	13.0	11.9
37.....	56.0	13.7	24.5	73.....	111.5	12.0	10.8
39.....	60.0	13.7	22.8	75.....	114.0	10.2	9.0
41.....	63.5	13.7	21.6	77.....	115.5	8.2	7.1
43.....	67.0	13.5	20.2	79.....	116.5	5.5	4.7
45.....	70.5	13.3	18.9	81.....	118.0	2.5	2.1
47.....	74.5	13.2	17.7	83.....	119.5	0.0	0.0

* Length of pulp in relation to total length. The measurements are the average of 5 plucked feathers for each entry.

† The sum of all measurements of length of pulp, amounting to 430.6 mm., is referred to on p. 165.

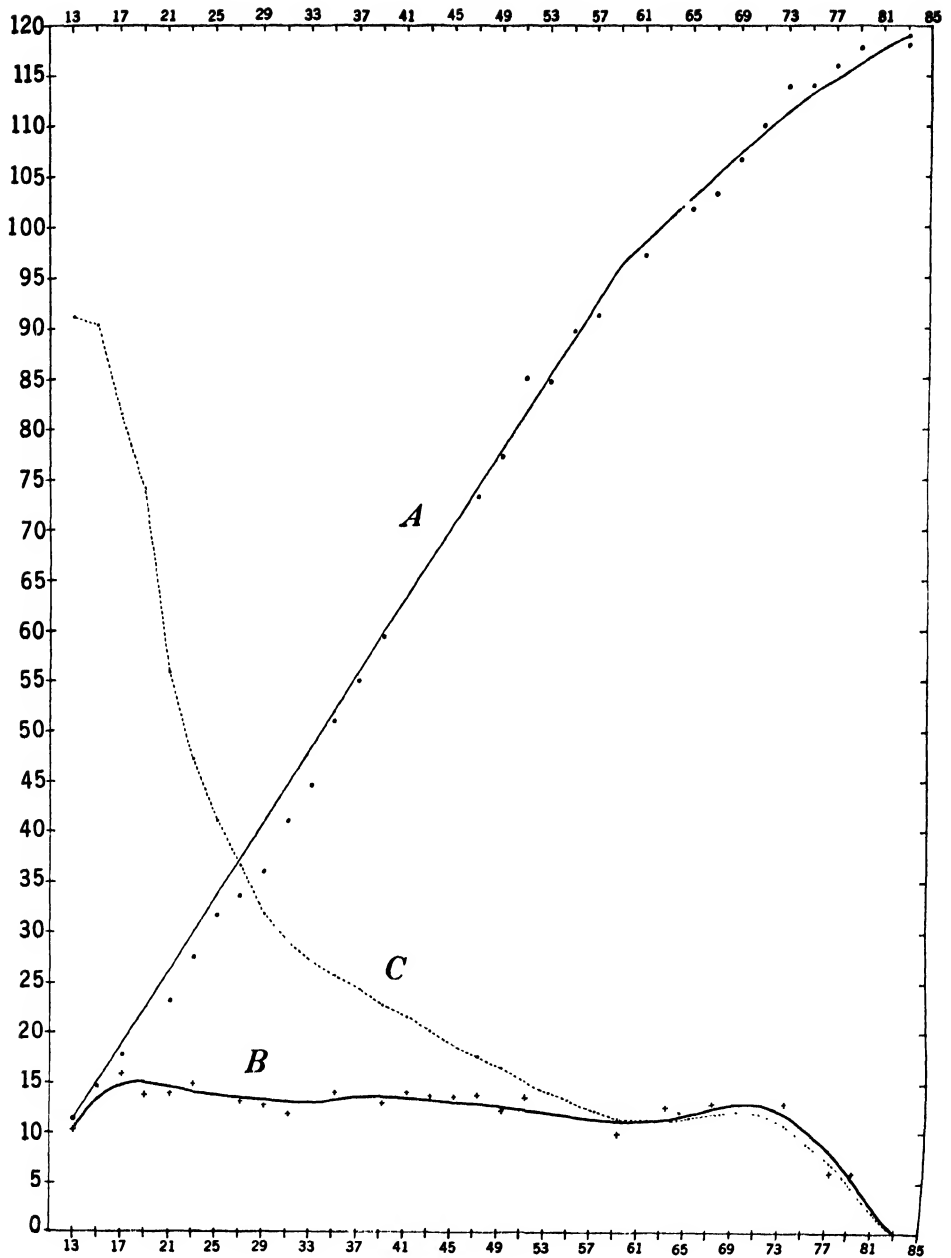


FIG. 3.—Curves of growth, feather and pulp, saddle feather, Brown Leghorn capon 148. Data from Table 4. *A* = feather; *B* = pulp; *C* = percentage of length of pulp to length of entire feather. Abscissae = days of regeneration; ordinates = length in millimeters for curves *A* and *B*, percentages for curve *C*.

on Table 4, however, agree very well with one another and are the same in principle as for the breast feathers of the White Leghorn. It will be noted that the measurements begin on the thirteenth or the fourteenth day of regeneration. The reasons for this will appear later.

The curves of growth of the entire feather do not differ significantly from previous determinations (Juhn, Faulkner, and Gustavson, 1931; Lillie and Juhn, 1932). The curves of length of the pulp and of percentage of length of pulp to the entire feather are, however, new determinations. The outstanding feature is that the pulp attains a certain "functional" length at about the fourteenth day of regeneration in the case of the breast feather, and a little later in the case of the saddle feather, which is maintained with only slight fluctuations throughout most of the period of regeneration. In the last 10-12 days of regeneration (cf. tables and curves for details) the length of the pulp decreases rapidly until it is all gone. This coincides with the period of most rapid decrease of rate of growth of the entire feather, and agrees approximately with the period of formation of the calamus of the feather.

2. FIXED RELATIONS OF PULP AND FEATHER

Considered entirely by themselves, these figures and curves, especially the long "plateau" in the case of the length of the pulp, might suggest that the ectodermal component of the cylindrical growing part of the feather is constantly slipping over the pulp in its apparently more rapid growth. The consideration that this would involve constant disturbance of delicate nutritive relations suggests doubts which are reinforced by the intimacy of relations between the pulp and the growing part of the feather. The relationship of the pulp to the ectodermal cylinder is so close that it is difficult to strip the pulp from the opened cylinder without carrying with it parts of the ridges, containing large melanophores in the case of black feathers. This is true in certain breeds of fowl, at least.

The alternative theory would be that the relations between the pulp and all formed loci of the ridges are permanently fixed throughout growth; that the pulp accordingly grows at the same rate, to the same extent, and in the same dimensions as the ectodermal component; and, therefore, that the total growth in length of the pulp during regeneration is equal to that of the feather itself. From this it would follow that the functional length of the pulp, as given in the curves, is regulated by resorption at its apex, where it becomes free from the shaft and barbs of the growing feather. We discussed these relations previously (Lillie and Juhn, 1932, pp. 145-46) and concluded: "We must thus think of the pulp as constantly growing at its base, and constantly dying and being resorbed at its apex."

We have now tested these hypotheses experimentally, and also by study of the process of resorption which results in the formation of the "pulp caps."

During a period of 7 weeks about 100 regenerating breast feathers of Brown and also of White Leghorns were operated on as follows, and 77 useful results were obtained. The procedure was to perforate a growing feather of from 21 to 28 days' regeneration transversely by a fine wire; in some cases the ends were cut off short and a piece left in; in others the wire was used to pull through a delicate silk thread, stained or unstained, a piece of which was similarly left *in situ*; finally, in yet other cases, a thread stained in gelatin carmine or Janus green was drawn completely through, leaving a stained track. At the stage of operation the pulp is 14-15 mm. long, approximately

8 mm. of which is above skin level. The perforation was made at skin level, and the track passed through sheath and barbs, through the center of the pulp, and out on the opposite side. Most of the operated feathers survived and grew at a normal rate, except for a possible brief period of inhibition following the operation.

The point to be ascertained was whether the pulp grew at the same rate as barbs and sheath—especially, of course, with reference to maintenance of fixed relations between the periphery of the pulp and the ectoderm. The experiments were uniformly convincing on this point. The mark on the periphery of the pulp and the external scar were always in perfect alignment.

The first experiments were with an exceedingly fine wire, left in place with free ends on the two sides. These experiments were exploratory with a view to ascertaining the possibility of the method. Objection could be raised to the use of the wire left in place on account of its rigidity. However, there was never any evidence, on careful examination by dissection, of strain in the pulp, such as would be expected if the rate of growth of the pulp were markedly different from that of the ectodermal cylinder. Seventeen feathers were so operated, and observations were made on different feathers 24, 48, and 72 hours after insertion. The rate of growth of the entire feather was 2.5–3.0 mm. per day, and the wire moved an equal distance above the surface of the skin; thus, after 3 days, as the pulp maintains an approximately equal length during this period, the wire was near the tip of the pulp, or beyond it, in a forming pulp cap. In all of the cases in which the wire perforated both sides of the sheath there was no evidence on dissection of abnormal relations of pulp and feather. Both appeared to have grown at the same rate.

In some cases in which the wire was purposely not pushed through the opposite side, the free end in the pulp was at a slightly lower level than the opposite end perforating the sheath, thus, as a whole, being in a slightly slanting position. This suggested that the center of the pulp might be growing at a slightly slower rate than the periphery. It was, however, noticeable that the slant was as pronounced 24 hours after insertion of the wire as it was later.

The remaining experiments with fine silk threads or stained tracks were made to test the results with the wire left in place. Without exception they demonstrate that the surface of the pulp grows at the same rate as the sheath and barbs, for the points of entry on both sides exactly correspond even after 48–72 hours' growth (Fig. 6). The conclusion is then unescapable that the relation of the pulp and ectodermal component is a fixed one, at least for the portion above the skin. The same conditions must hold also for the portions yet in the follicle, which are constantly emerging from it as growth progresses.

The apparent slight difference between growth in the center of the pulp and at the

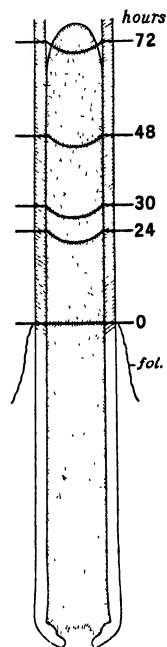


FIG. 6.—Diagram of path of track made by a silk thread left *in situ* from the time of its insertion through the center of the pulp. At the surface of the skin = 0; 24, 30, 48, and 72 show the position and the form of the track at 24, 30, 48, and 72 hours after insertion. \times ca 9.

periphery remains to be explained. The course of the fine silk thread or of the stained track in the pulp is usually somewhat curved downward from periphery to center of pulp; but this is variable, and the path is sometimes approximately straight. Moreover, the curve is not more pronounced at 48 or 72 hours after insertion than at 24 hours (Fig. 6). The insertion of the wire of course ruptures some of the delicate-walled blood vessels of the pulp, and there may be slight external bleeding. The large vessels, especially the axial artery, are toward the center of the pulp, and hence the greatest injury is here. The evidence indicates that the downward curve in the track is an injury phenomenon, and that, once repaired, growth is equal from center to periphery of the pulp.

These experiments also demonstrate the occurrence of resorption at the apex of the pulp. Any transfixing level of the pulp becomes apical in the course of growth, and then disappears, leaving the transfixing wire or thread free above it. Thereafter the wire or thread continues to be carried upward by the continued growth of sheath and barbs.

3. RESORPTION AND THE PULP CAPS

A. INTRODUCTION

The resorption of the pulp takes place apically; it follows from the principle of equal growth of pulp and feather that the total amount of resorption is measured in length at any time recorded in the curves by the vertical distance that separates the curve of length of the pulp and of the entire feather (Figs. 2 and 3).

Before about the fifteenth day of regeneration typical pulp caps are not formed, and the length of the pulp is constantly increasing.⁵ Thereafter the amount of resorption is approximately equal to the growth, as shown by the plateau form of the curve of length of the pulp for a long time during regeneration; finally, resorption overbalances growth, and the entire pulp disappears with the completion of regeneration.⁶ The actual length of pulp at any time depends upon the relation between growth and resorption. The capillary system of the pulp is specially developed apically in connection with resorption (see sec. IV).

The growth of the pulp is continuous, but resorption is periodic and accompanied, after about the fifteenth day, by the formation of "pulp caps" at the rate of about one per day. In order to understand this process we must first consider the ectoderm membrane (stratum cylindricum) that separates the pulp from the formed elements of the feather.

B. THE STRATUM CYLINDRICUM

The ectoderm of the feather germ early becomes differentiated into three layers (Pl. I, Figs. 4 and 5): an outer layer (stratum corneum) next to the cavity of the follicle from which the sheath is derived; a thick middle layer (stratum intermedium)

⁵ The formation of pulp caps is always associated with resorption of pulp, as described below. In the case of breast feathers, the first pulp cap is usually found on the sixteenth day of regeneration and only exceptionally as early as the fifteenth day. In the case of saddle feathers, the first pulp cap is formed between the fourteenth and sixteenth days. For earlier history of the pulp and the beginning of resorption see sec. III, 4.

⁶ The rate of resorption is, however, not greatest at the end. Between the twenty-sixth and twenty-eighth days of regeneration in the case of the anterior breast feathers, 5 mm. of pulp was resorbed during the plateau period; between the forty-second and forty-fourth days during the period of diminution in length of the pulp, only 2 mm. of pulp was resorbed (see Table 1).

from which the entire feather—barbs, barbules, and shaft—is derived; and an inner epithelial layer (*stratum cylindricum*), next to the pulp. These layers are united basally in the collar or formative epidermal ring surrounding the umbilicus of the feather germ, from which they continue to be derived during regeneration (Pl. II, Fig. 1; and text Figs. 18 and 19).

In descriptions of the development of the feather attention has been directed naturally to the middle layer. The inner layer has been regarded as forming only "accessory structures," and its development has been relatively neglected in recent times, although by no means overlooked by earlier students of feather development (cf. Davies, 1889). At all stages it intervenes between the pulp and the feather. It is very important with reference to the functions of the pulp and therefore requires careful study.

Near the base of the feather germ the *stratum cylindricum* is composed of a single layer of cubical or cylindrical epidermal cells completely covering the pulp. It clothes the surfaces of each ridge, both centrally, i.e., toward the pulp, and also laterally, i.e., between apposed faces of the ridges (Pl. I, Fig. 5). At the peripheral or outer end of each ridge as seen in cross-section, it extends to the inner layer of the sheath and bends back over the lateral surface of the adjoining ridge.

The *stratum cylindricum* is thus a single membrane folded in between the ridges; but, with reference to the subsequent development, the two adjacent layers belonging to neighboring ridges will be designated as "barb septa." Each of the latter thus consists of two apposed layers; like the ridges themselves, the barb septa terminate at the rhachis, and they extend between adjacent ridges to their tips. The remainder of the *stratum cylindricum*, on the other hand, clothes the surface of the pulp directly and completely; it is thus continuous over the surface of the rhachis and, on the opposite side, over the tips of the ridges. It faithfully molds the contours of the ridges and rhachis, so that, when ultimately separated from them after keratinization, it bears their impressions permanently (Pl. III, Figs. 12 and 13).

In an apical direction the part of the *stratum cylindricum* next to the pulp becomes a stratified epithelium in which two principal layers may be distinguished—an external single layer of squamous epithelium, the "barb membrane," and the remainder next to the pulp, which may be known as the "pulp membrane." Over the apex of the pulp the latter becomes specialized as the "apical membrane of the pulp."

In summary, then, the *stratum cylindricum* differentiates apically into three parts, viz.: the barb septa, the barb membrane, and the pulp membrane.

C. RESORPTION OF THE PULP

Resorption of the pulp is periodic, and each period of resorption is followed by a period of reconstitution of the pulp and the apical membrane. We distinguish, for purposes of description, three stages of resorption, numbered "I," "II," and "III," and two stages of reconstitution, numbered "IV" and "V." Together, these stages cover the complete cycle of formation of a single pulp cap. Each stage is characterized by typical changes of the apical membrane and of the pulp itself, which are presented in the accompanying diagrams (Fig. 7).

We may start the description with stage V, which represents the completion of reconstitution and immediately precedes stage I of resorption. Three completed caps are represented above the apex of the pulp. It will be understood that the parts shown are completely wrapped within the yet unfolded feather. Three layers are represented in

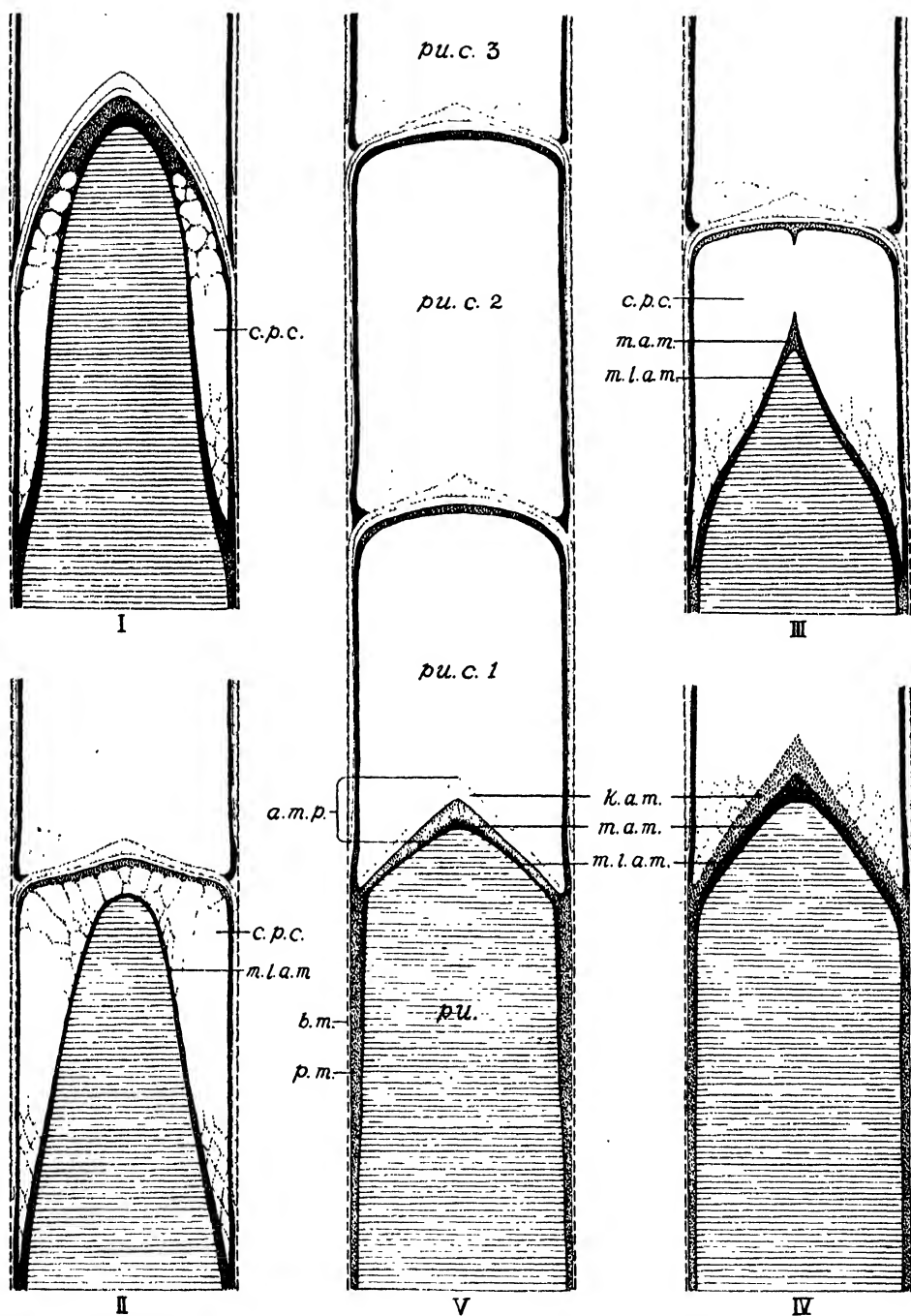


FIG. 7.—Diagrams of the five stages of pulp-cap formation described in text. See table of abbreviations, p. 175.

the ectoderm below the apex of the pulp. The thin external layer represented by a broken line is the barb membrane not concerned in the process of resorption; within this the pulp membrane exhibits two layers which are concerned in periodic processes of resorption, viz.: the Malpighian layer and a thick middle layer.

Over the apex of the pulp it will be noted that the middle layer has formed two layers—an external one, composed of curious keratinized but not flattened cells (cf. Pl. II, Figs. 8, 9, and 10), destined to form the main thickness of the dome of each cap; and an internal one, not further differentiated.

Resorption, of course, concerns the pulp itself; and as it proceeds, the pulp shrinks, first in diameter and then in length, in a manner that can be followed through stages I, II, and III. While this is going on, changes in the ectoderm occur that lead to the production of the cavity of the pulp cap. This cavity arises in the middle layer of the pulp membrane as a large number of vacuoles which gradually run together so as to leave an undivided space. This happens first about the middle of the future pulp cap, and later the cavity extends within the apical membrane itself, so as to separate the pulp completely from the dome of the forming pulp cap (stages II and III).

The pulp is thus left with a thin covering of Malpighian cells and a minimal portion of the middle layer of the pulp membrane. The Malpighian layer then rapidly reconstitutes the apical membrane, as shown in stage IV. In stage V the keratinized layer of the apical membrane is reformed; and, together with its branching extensions, it constitutes the dome of each pulp cap, as shown in Plate III, Figure 13.

The pulp cap, thus formed, has an arched dome consisting of two layers, but the sides include also the barb membrane and are thus three-layered. The diagram greatly exaggerates the thickness of the sides of the pulp cap (cf. Pl. II, Figs. 8, 9, and 10). Subsequently, as the drying-out is completed, the sides become broken off from the dome of the succeeding cap (Pl. III, Fig. 13, *B*), and each cap is then a little thimble of keratin.

These phenomena have not hitherto been described, and they accordingly deserve fuller illustration. We present in Plate II, Figures 8, 9, and 10, photographs of longitudinal sections corresponding to stages I, II, and IV of the diagram. Study of the sections and their captions will serve to correct the account just given, so far as it is diagrammatic.

In the formation of the calamus the same process, periodic even during the final resorption of the pulp, gives rise to a series of "horny" partitions, known of old. The Germans have the curious name "Federseele" for these structures. The term "scala of the calamus" has also been applied to them collectively.

The changes in the pulp that accompany its resorption are conditioned by the highly developed terminal arborization of the axial artery (text Fig. 18; Pl. IV, Figs. 22 and 23). Figures 8, 9, and 10 of Plate II show the histological pictures for stages I, II, and IV. The apical vessels lose their walls (Pl. II, Figs. 8 and 9), and the blood corpuscles become free in the pulp. These pictures do not, of course, reveal either the sequence or the nature of the events. It is possible that there is a preliminary leucocytosis, but the first action may be autolytic. Presumably the second step is an extensive phagocytosis followed by a restoration of the products of resorption to the blood stream. Reconstitution of the axial artery is to be seen in Plate II, Figure 10 (stage IV), and also diminution of blood corpuscles in the pulp.

In some cases resorption of the axis of the pulp is incomplete—exceptionally in the case of the breast and saddle feathers of the White Leghorn, and quite regularly in the

case of the wing primaries. Under these circumstances a central strand containing haemoglobin and presumable remains of the axial artery runs through the axes of successive caps, binding them together in the manner shown in Plate III, Figure 11.

D. THE PULP CAPS

The pulp caps thus formed are little thimbles of keratin with thickened domes provided with branching processes, as shown in Plate III, Figure 13. The series of caps is at first inclosed by the special barb membrane represented by the broken line in the diagrams (Fig. 7); to this the keratinized barb septa are loosely attached. Figure 12 of Plate III shows the barb septa still in place on the caps. In this figure, *A* is oriented to show the deep impression of the rhachis in the center (the barb septa begin abruptly here in their half-spiral around the cylinder); *B* shows the rhachis groove on the left and exhibits the complete course of some of the septa; and *C* is another view similar to *A*. The septa are lost very quickly as the feather unfolds, but the barb membrane itself

No.	Total Length (Mm.)	Length of Pulp (Mm.)	Length of All Caps (Mm.)	No. of Caps	Average Length of a Cap (Mm.)	Tip Free from Caps (Mm.)
33 Days' Regeneration						
1.....	49	12	34	19	1.79	3
2.....	48	12	32	20	1.60	4
3.....	50	12	34	22	1.55	4
4.....	46	13	29	18	1.61	4
5.....	50	13	33	21	1.57	4
Av.....	48 6	12 4	32 4	20	1 62
37 Days' Regeneration						
6.....	54	15	38	24	1.60	2

remains adherent to the sides of the caps. Plate III, Figure 13, shows dried pulp caps after loss of the barb ridges.

In the regenerating feathers examined by us, a number of complete pulp caps, varying from one to about five, are usually found beyond the apex of the pulp, wrapped in the barb membrane within the incompletely unfolded barbs. As the unfolding of the barbs is completed, the caps become detached and are lost. It is therefore impossible to determine in the ordinary feather, by count with any considerable degree of accuracy, how many caps are formed in a given period of regeneration of the feather. However, we were fortunate enough to find a number of regenerating saddle feathers of known age in a Brown Leghorn capon in which the sheath of the feather was persistent nearly to the tip (Pl. III, Fig. 14); the feather had therefore not unfolded, and all the pulp caps were retained and could be readily counted.⁷ Five of these were plucked on the thirty-

⁷ Samuel (1870), in a paper designed for study of the influence of nerves on growth and regeneration of feathers, found that, when the innervation of the wing of the pigeon is severed, the sheath of regenerating feathers persists to the tip, and that they thus appear like long spines. He does not regard this result

third day of regeneration, and the only remaining one with complete sheath was plucked on the thirty-seventh day. The measurements and number of caps in these feathers are given in the table on page 156.

As noted previously, the first cap in the case of saddle feathers of Brown Leghorn capons forms on about the fifteenth day of regeneration. This is subject to some variation, depending on the length of the period of quiescence following plucking and presumably on some other factors; but the amount of variation does not appear to exceed a day usually, or rarely 2 days. In the case of the feathers 33 days of age, the period of cap formation would thus last about 18 days, and at 37 days it would last approximately 22 days. The average number of caps formed in the group of five 33-day feathers is 20, and in the 34-day feathers it is 24, in both cases close to the presumed number of days of cap formation; and the average length of a cap, 1.62 mm., corresponds quite closely to the average daily increment of length of saddle feathers of the same age (cf. Tables 3 and 4).

It was natural, therefore, to suspect at first the existence of a diurnal rhythm. However, in the group of 33-day feathers the number of caps ranges from 18 to 22, which, on such an assumption, would appear to fall a little outside the expected range of variation in time of formation of the first cap. Moreover, if the individual lengths of caps are examined, more variation is found than is consistent with the very regular daily rate of growth of the feather.

If there were a diurnal rhythm, one would expect to find that the various stages of cap formation, and particularly the occurrence of periods of resorption and reconstitution, would tend to be at definite periods of day and night. Accordingly, this possibility, which would be of considerable interest, was carefully examined.

Regenerating breast feathers of from 18 to 25 days of age were plucked at 6:00 A.M., 12:00 M., 6:00 P.M., and 12:00 midnight in consecutive 24-hour periods *on two separate occasions*. Three Brown Leghorn capons were used, and about 12 feathers were taken from each capon at each time, making 288 in all; 277 of them were classified by the stages of cap formation, as shown in the accompanying table.

TIME	RESORPTION			RECONSTITUTION		TOTAL
	Stage I	Stage II	Stage III	Stage IV	Stage V	
6:00 A.M.....	10	11	11	17	15	64
12:00 M.....	14	14	11	14	25	78
6:00 P.M.....	15	12	7	14	19	67
12:00 midnight.....	7	11	12	20	18	68
Total.....	46	48	41	65	77	277
Grand total.....	135			142		277

as due directly to lack of innervation, but rather to the incidental effects of paralysis of the wing. The feathers of the paralyzed wing are never moved or preened. He accordingly attributes the normal loss of the sheath to a point a certain varying distance above the opening of the follicle to movements of the feathers themselves and to preening by the bird, an opinion in which we concur. However, we regard it as probable that other factors, such as abnormal thickness of the sheath, might be responsible for its retention in certain circumstances.

The stages of resorption and reconstitution collectively are approximately equal, and it is therefore probable that they occupy about equal periods of time. Since all stages are found at any hours of the day or night—and this has been confirmed by repeated, less systematic observations—it is necessary to conclude that the periodicity is not determined directly by the regular diurnal fluctuations in metabolic rate (Barott *et al.*, 1938). We must accordingly look for some other causes of the periodicity in this phenomenon.

The problem is obviously related to that of the determination of the length of the pulp, which is quite constant throughout the greater portion of the period of regeneration (Tables 1 and 2; Figs. 2 and 3). The maintenance of an approximate standard of length of pulp specific for different feather tracts indicates some function of length.⁸ We have also ascertained that, if apical portions of the pulp are cut off (breast feathers), the remainder tends to regain its original length in the process of further growth. The position that length of pulp has a functional significance is thus reinforced.

We previously postulated a relation between length of pulp and length of barbs (Lillie and Juhn, 1932). It is true that the length of pulp is usually greater than that of the longest barbs; and it may, as a matter of fact, be much longer in certain tracts. But even this does not appear to be invariably true.

The length of pulp bears a regular relationship to the normal length of the sheath, which extends a short distance beyond it, and to the position above the sheath where the barbs unfold and separate from the barb membrane and barb septa. But that this also is not a determining factor is shown by the cases in which the sheath is persistent almost to the tip of the feather, and the pulp caps are nevertheless regularly formed (Pl. III, Fig. 14).

The functional length of the pulp is regulated by the periodic process of cap formation, which involves both a limited amount of resorption of pulp and the accompanying differentiation and reorganization of the apical membrane of the pulp. In ordinary biological terms the function of the apical membrane is obviously to protect the vascular pulp containing 93 per cent of water (see p. 167) from desiccation. The pulp caps above the apical membrane and the thick wrapping of feather and sheath are admirably adapted to reinforce this protection.

It is also obvious, from a biological point of view, that resorption could hardly be a continuous process, like growth. Periodic reorganization of the protective organ after resorption would appear to be necessary in order that fatal exposure should not occur at any step. From the same point of view, admitting that a certain standard of length of pulp has functional significance, resorption cannot be allowed to proceed too far basally at any time, and this distance is actually related to the rate of growth; the most rapidly growing feathers have the longest pulp caps.

The cells of the collar, which is the source of the three layers of the feather germ, are rich in protoplasm, and many of them are in process of division; the collar must therefore contain a large percentage of water. The processes of differentiation progress in an apical direction in all three layers and result in completely desiccated keratinized derivatives. Cell division is found in the bases of the barbs and in the ventral membrane of the shaft for a short distance above the collar; in the case of the sheath, there is no

⁸ In the wing primaries the length of the pulp is three to four times greater than in breast or saddle feathers.

cell division above the collar; and in the case of the pulp membrane, multiplication of cells extends to the apex of the pulp. This is evidence that the processes of differentiation and of desiccation proceed at different rates in the three layers of the feather germ; but in all parts there is a gradient in cellular water content, briefly a water gradient, from the base of the feather germ in an apical direction.

The derived structures acquire rigidity according to the degree of keratinization and differentiation, which go hand in hand with loss of water; and it is obvious that, until the degree of rigidity assures independent maintenance of the form of the feather and the interrelations of its parts, it must have outside support and protection against bending and too rapid desiccation. It is probable that adequate rigidity is not attained much below the apex of the pulp, for bending below a certain point results in permanent deformation. While the shaft and barbs of the feather are still soft and pliable, they must be held straight and firmly in position. As the feather develops in the wall of a cylinder, it is necessary that the wall be smooth and of adequate dimensions. The pulp, by its turgor (p. 172), maintains the smooth form of the cylinder against the resistant sheath. It also keeps the surfaces of the barbs moist below the thickening of the apical third of the pulp membrane. The sheath always extends a considerable distance beyond the apex of the pulp, and within this distance the drying of the surfaces of the barbs appears to be completed before they unfold.

This result throws light on the water gradient, the functional length of the pulp, and on the role of the sheath. The functional length of the pulp would appear to be related to the length of the water gradient, and protection against desiccation is strongly emphasized as one of the chief functions of the sheath.

We can thus see clearly that the whole process of cap formation is very perfectly adapted to the requirement of a standard length of pulp, but we do not yet fully understand the nature of this requirement or the physiological mechanisms of the periodicity by which it is regulated.

E. HISTORICAL

There is curiously little information in the literature about the dermal components of the feather germ. Prior to the paper by Davies (1889), which marks a turning-point in our knowledge of development of the feather, the observations are scattered and quite incidental. The pulp was early recognized as the nutritive organ of the feather germ, and it was understood that it underwent complete resorption after the feather was fully formed. The pulp caps were seen even by Malpighi (1697), who derived them from dried-up apical portions of the pulp. Others who referred to the pulp caps were Dutrochet (1819) and Burmeister (1840). The scala in the calamus was early recognized and described. Samuel (1870) made the first experiments on the pulp—indeed, the only ones to date.

Davies (1889), who had a remarkably orderly mind, was the first to state clearly the fundamental principles on which subsequent interpretation has rested. The pulp grows from its base and is resorbed at its apex;⁹ neither the process of resorption nor its rate was described in detail, but he connected the formation of pulp caps with the process of resorption and derived them correctly from the stratum cylindricum. He did not, however, observe the apical membrane of the pulp, and postulated, instead, periodic contractions and concomitant thickening of the stratum cylindricum over the apex of

⁹ Hosker (1936, p. 164) erroneously ascribes to Davies her own mistaken view that the pulp is resorbed at the base.

the resorbing pulp. He also implied that the scala in the calamus is similarly formed. He derived the barb septa also from the stratum cylindricum.

The subject has rested in about the form that Davies gave it down to the present time. Hosker (1936), in her very comprehensive paper, devotes some attention to the pulp and the pulp caps. She states:

As the pulp passes down the feather, the cylinder cells become congested, and the outermost ones, being further from the nourishment contained in the abundant blood supply of the pulp, are overtaken by the process of cornification. This occurs at the sides as well as the tip of the pulp, fig. 37, Plate 22, so that a series of cones is formed, connected with each other.

In this way the feather caps arise, and, where protected by the calamus, persist, although distal to the superior umbilicus they soon break away from the feather.¹⁰

The figure referred to is a drawing quite similar to our photograph (Pl. II, Fig. 9), but it is misinterpreted, owing to her mistaken opinion that the pulp is resorbed at the base.

4. EARLY HISTORY OF THE PULP

The measurements of the relative lengths of pulp and feather in breast and saddle recorded in Tables 1-3 and in the curves in Figures 2 and 3 begin on the thirteenth and fourteenth days of regeneration. At this time the regenerating feather has emerged from the mouth of the follicle, and the tip of the originally closed sheath has been lost, so that a tuft of barbs extends beyond the sheath. Before this time the sheath is closed over the tip of the feather; hence the common term "pinfeather."

Growth during the pinfeather stages exhibits certain special features. It is decidedly slower during about the first 9 days, while still concealed in the follicle, than later (Lillie and Juhn, 1932). This period is concerned with the primordial organization of the feather germ, during which the elements of the tip of the feather are laid down.

During the same period the sheath and the pulp are much longer than the feather proper (Fig. 15); the stratum intermedium is absent over the upper portion of the pulp; and the stratum corneum (sheath) and the stratum cylindricum (pulp membrane) are in immediate contact here. The sheath is thick and strongly keratinized, and the pulp membrane has developed a special apical membrane over the tip of the pulp.

At about the eighth day an apical cavity (Fig. 15, *a. c.*) begins to appear between the tip of the pulp and the sheath, which rapidly increases in length until the loss of the closed apex of the sheath, when it opens to the exterior between the tuft of apical barbs. Measurements show that the distance from the dorsal barbs to the tip of the sheath (Fig. 15, 1 + 2) remains quite constant until the loss of the sheath, about the thirteenth day; during this time the total length of the feather germ (Fig. 15, 1) has increased from approximately 3 mm. (eighth day) to 9.5 mm. That is to say, the feather has grown during this period, as also later, at the same rate as the sheath. On the other hand, the distance between the barbs and the apex of the pulp (Fig. 15, 2) diminishes steadily during the same period, and the barbs come to exceed the pulp in length. Simultaneously, the length of the apical cavity (Fig. 15, 1) increases *pari passu*. The latter facts are to be explained by resorption of the pulp at its apex,¹¹ which begins about the eighth day, resulting in the formation of the apical cavity. Between the eighth and

¹⁰ Hosker, 1936, p. 163.

¹¹ The typical pattern of circulation with special provision for resorption of the apex of the pulp has developed by, or before, the ninth day of regeneration (cf. p. 173).

the thirteenth days, however, the rate of growth of the pulp exceeds the rate of resorption, so that a net increase in length results until the functional length of the pulp is attained (cf. Figs. 2 and 3). We therefore reach the conclusion that, after the feather germ is once organized, the pulp and the ectodermal components grow at the same axial rate, as we have shown to be the case in later stages.

Resorption of the pulp is periodic during the pinfeather stages. As in later stages, the apical membrane exhibits successive periods of keratinization of its outer cells, followed by splitting-off. To this extent the process resembles the formation of pulp caps in later stages; but the partitions across the apical cavity thus resulting, corresponding to the domes of the typical pulp caps, are relatively very delicate, and proper pulp caps are not formed.

5. THE WEIGHT OF THE PULP COMPARED WITH THE FEATHER

The preceding section deals with the linear dimensions of the pulp. The present section presents our data on weight of pulp produced.

In order to determine weights, it is necessary to pluck the feather, and this precludes the possibility of using the same feather for different ages of regeneration. The age comparisons are, therefore, those of different feathers, and this introduces sources of error that it is impossible to eliminate entirely. We have sought to reduce them as much as possible in the following ways: (1) by using the same bird for a complete set of determinations and keeping the records for different birds separate, (2) by using only feathers of known location and regeneration age, and (3) by averaging for each stated figure the results on 5 neighboring feathers of the same bird (in the case of saddle feathers).

In spite of the difficulties, consistent results have been obtained. This comes out strikingly in the comparison of weights of constituents in the saddle feathers of two capons given beyond, which give practically identical results. The weights determined are those of the entire plucked feather; of the feather after removing the pulp; and of the pulp itself, calculated as the difference between the first two (Tables 5 and 6). The data of Table 5 are plotted in the graphs of Figure 16.

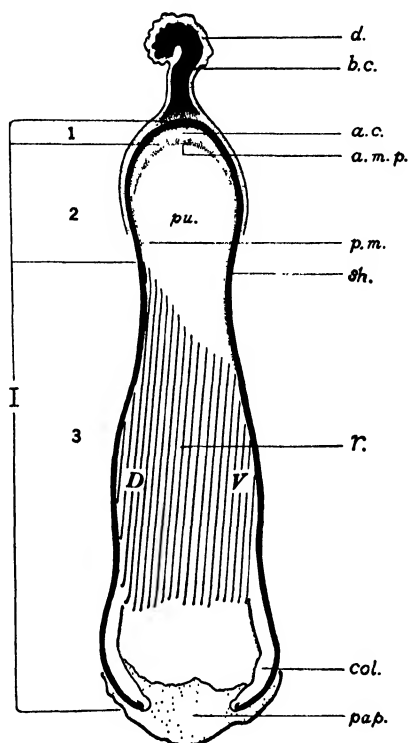


FIG. 15.—Feather germ of 8 days' regeneration from the breast, Brown Leghorn capon, dissected out of the follicle. Entire preparation from the right side; the dorsal surface (*D*) is to the left and the ventral surface (*V*) to the right. The tip of the preparation bears a blood coagulum formed as a result of plucking, inclosed within debris from the wall of the follicle. *I* = total length of feather germ; 1 = extent of the apical cavity; 2 = length from the dorsalmost barb to the tip of the pulp; 1 + 2 = distance from the dorsalmost barb to the apex of the sheath; 3 = distance from the umbilicus to the tip of the dorsalmost barb. \times ca. 25.

A. SADDLE FEATHERS (CAPONS 171 AND 148)

Practically the entire saddle region was plucked simultaneously in each bird on December 28, 1938. Beginning on the fourteenth day of regeneration (capon 171) or the thirteenth day (capon 148), the weights given in Tables 5 and 6 were determined on

TABLE 5
BROWN LEGHORN CAPON 171: SADDLE FEATHER*

Age (Days)	Total Weight (Cgm.)	Weight of Feather (Cgm.)	Weight of Pulp (Cgm.)†	Percentage of Pulp Weight to Total Weight
14.....	17.0	8.0	9.0	52.9
16.....	26.0	13.0	13.0	50.0
18.....	34.0	16.0	18.0	52.9
20.....	40.0	19.5	20.5	51.2
22.....	46.0	22.7	23.3	50.7
24.....	49.5	25.5	24.0	48.5
26.....	53.0	28.2	24.8	46.8
28.....	56.0	30.2	25.8	46.1
30.....	58.2	32.5	25.7	44.2
32.....	60.0	34.5	25.5	42.5
33.....	60.7	35.4	25.3	41.7
35.....	62.0	37.0	25.0	40.3
37.....	63.5	38.5	25.0	39.4
39.....	64.3	40.0	24.3	37.8
41.....	65.0	41.2	23.8	36.6
43.....	65.7	42.2	23.5	35.8
45.....	66.4	43.0	23.4	35.4
47.....	67.0	43.3	23.6	35.2
49.....	67.5	44.0	23.5	34.8
51.....	67.7	44.5	23.2	34.4
53.....	68.0	45.0	23.0	33.8
55.....	68.2	45.5	22.7	33.3
57.....	68.3	46.2	22.1	32.4
59.....	68.4	46.7	21.7	31.7
61.....	68.3	47.5	20.8	30.5
63.....	68.3	48.0	20.3	29.7
65.....	68.1	49.0	19.1	28.0
67.....	68.0	50.0	18.0	26.5
69.....	67.7	51.0	16.7	24.7
71.....	67.5	52.5	15.0	22.2
73.....	67.0	54.0	13.0	19.4
75.....	65.5	55.0	10.5	16.0
77.....	63.6	55.7	7.9	12.4
79.....	61.5	56.7	4.8	7.8
81.....	58.7	55.7	3.0	5.1
83.....	56.0	55.0	1.0	1.8
85.....	54.0	54.0	0.0	0.0

* Weight of pulp in relation to total weight of feather. Total weight of 5 feathers, multiplied by 2, entered in centigrams. The same feathers which were used for length determinations in Table 3 were used here.

† The sum of all determinations of weight of pulp, amounting to 690 cgm., is referred to on p. 165.

alternate days in each case to the completion of growth of the feathers. (Note that after the thirty-second day the weighings were made on the same day for both birds.) Five closely placed regenerating feathers were plucked each time between 8:00 and 9:00 A.M. and weighed together; this weight, in centigrams, multiplied by 2, is entered as total weight, which is therefore ten times the average weight of the 5 feathers. No atten-

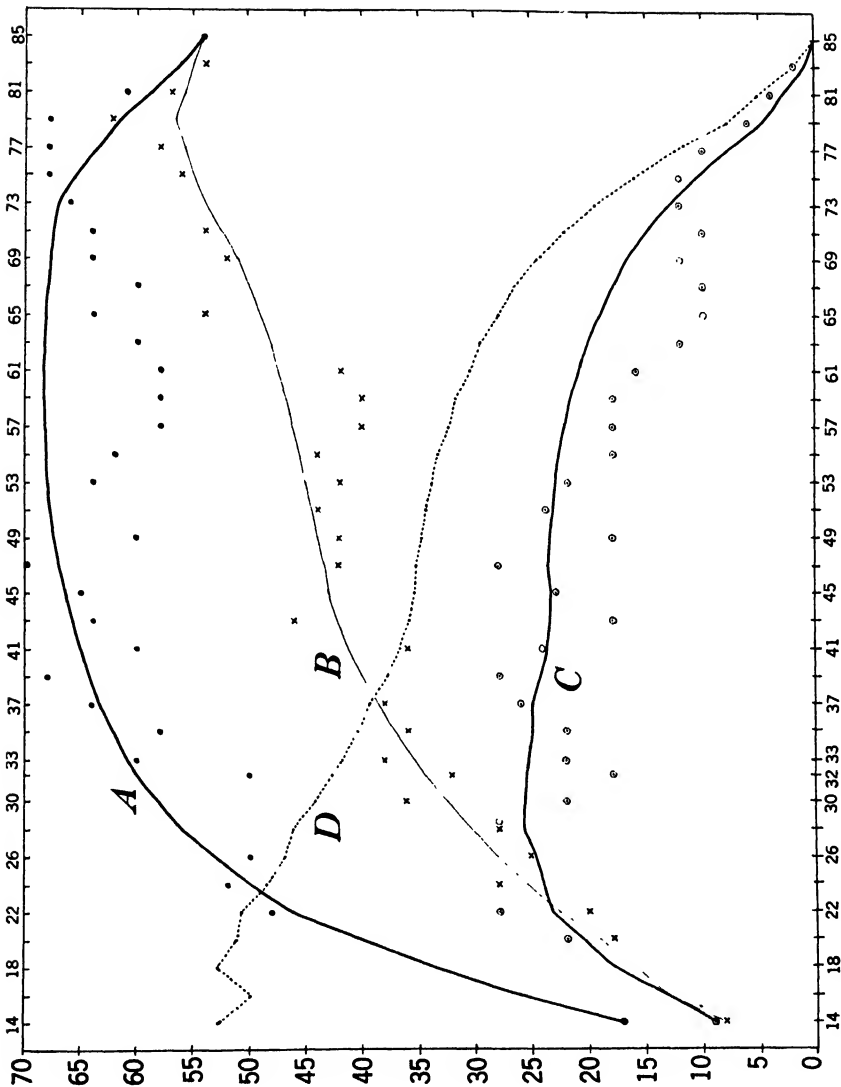


FIG. 16.—Curve of weights of constituents of regenerating saddle feathers, Brown Leghorn capon 171. Data from Table 5. *A* = total weight; *B* = weight of feather; *C* = weight of pulp; *D* = percentage of weight of pulp to total weight. Abscissae = days of regeneration; ordinates = centigrams for curves *A*, *B*, and *C*, percentages for curve *D*. Weight is ten times the weight of a single feather.

tion was given to position in the entire field of regenerating feathers in successive pluckings, and this may be one of the sources of irregularity in the weight curves.

The pulp was then carefully removed, and the 5 feathers were weighed together again. This weight, multiplied by 2, is the weight of the "feather" given in the third

TABLE 6
BROWN LEGHORN CAPON 148: SADDLE FEATHER*

Age (Days)	Total Weight (Cgm.)	Weight of Feather (Cgm.)	Weight of Pulp (Cgm.)†	Percentage of Pulp Weight to Total Weight
13.....	16.0	10 0	6.0	37.5
15.....	26.0	13.0	13 0	50.0
17.....	35.0	16 5	18.5	52 9
19.....	42.5	20 0	22.5	52.9
21.....	48.0	22.5	25.5	53.1
23.....	52.5	25 0	27.5	52.4
25.....	55.0	27 0	28 0	50.9
27.....	58.0	29 0	29.0	50.0
29.....	60 0	30.0	30 0	50.0
31.....	62.0	31 2	30.8	49.7
33.....	63.5	32 5	31.0	48.8
35.....	65.0	34 0	31 0	47.7
37.....	66 0	35.0	31.0	47.0
39.....	67 5	36 0	31.5	46 7
41.....	68.2	37 5	30.7	45.0
43.....	68.8	39.0	29 8	43.3
45.....	69.1	40.0	29.1	42.1
47.....	69.5	41.5	28 0	40 3
49.....	69.6	42.5	27.1	38.9
51.....	70.0	43.5	26.5	37.9
53.....	70.0	44.5	25.5	36.4
55.....	69.8	45.5	24 3	34.8
57.....	69.6	46.5	23.1	33.2
59.....	69 5	47.0	22 5	32.4
61.....	69 0	47 5	21.5	31.2
63.....	68.5	48.0	20.5	30.0
65.....	68.0	48.5	19 5	28.7
67.....	67.5	49 5	18.0	26.7
69.....	66.5	50 0	16.5	24.8
71.....	65.5	51.0	14.5	22.1
73.....	64.0	51.7	12.3	19.2
75.....	61.5	52.5	9.0	14.6
77.....	59.5	51.7	7.8	13.1
79.....	50.0	51.0	5.0	9.0
81.....	53.0	50.5	3.5	6.6
83.....	50.0	50.0	0 0	0.0

* Weight of pulp in relation to total weight of feather. Total weight of 5 feathers, multiplied by 2, entered in centigrams. The same feathers which were used for length determinations in Table 4 were used here.

† The sum of all determinations of weight of pulp, amounting to 670 cgm., is referred to on p. 165.

column. The weight of the pulp is then entered as the difference between the total weight and the weight of feathers, and the percentage weight of the pulp is calculated. These feathers are the same ones that were used for determination of the total length and the length of pulp given in Tables 3 and 4. In capon 171 there were 185 feathers used in all, and 180 in capon 148.

From the figures of length and weight given in Tables 3 and 5 (capon 171) and in Tables 4 and 6 (capon 148) the following calculations may readily be made:

1. The average weight of 1 mm. of pulp during the entire period of regeneration is $69 \text{ cgm.} \div 443.3 \text{ mm.} = 0.155 \text{ cgm.}$ in the case of capon 171, and $67 \text{ cgm.} \div 430.6 \text{ mm.} = 0.155$ in the case of capon 148; thus the same in both.

2. The total weight of pulp produced for each feather during regeneration is $129 \times 0.155 = 20 \text{ cgm.}$ in the case of capon 171, and $119.5 \times 0.155 = 18.5 \text{ cgm.}$ in the case of capon 148.

TABLE 7

WHITE LEGHORN COCK: ANTERIOR BREAST FEATHER*

Age (Days)	Total Weight (Cgm.)	Weight of Ectodermal Component (Cgm.)	Weight of Pulp (Cgm.)†	Percentage of Pulp Weight to Total Weight
13.....	27.4	13.9	13.5	49.3
14.....	28.0	14.0	14.0	50.0
16.....	31.2	15.2	16.0	51.3
18.....	35.5	17.5	18.0	50.7
20.....	40.0	20.0	20.0	50.0
22.....	43.3	22.5	20.8	48.0
24.....	46.0	25.0	21.0	45.7
26.....	48.0	27.0	21.0	43.8
28.....	50.8	30.0	20.8	40.9
30.....	53.5	33.0	20.5	38.3
32.....	55.8	35.5	20.3	36.4
34.....	58.0	38.0	20.0	34.5
36.....	61.9	42.5	19.4	31.3
38.....	63.1	45.0	18.1	28.7
40.....	62.2	47.0	15.2	24.4
42.....	60.5	48.5	12.0	19.8
44.....	54.	46.2	8.0	14.8
46.....	48.5	44.5	4.0	8.3
48.....	44.8	42.5	2.3	5.1
50.....	42.4	41.0	1.4	3.3
52.....	42.1	41.0	1.1	2.6
54.....	41.0	41.0	0.0	0.0

* Weight of pulp in relation to total weight of feather recorded for a single feather in each entry; each entry selected to correspond as closely as possible to the feathers measured for length in Table 1.

† The sum of all determinations of weight of pulp, amounting to 307.4 cgm., is referred to on p. 166.

3. The weight of 1 feather produced during regeneration in the case of capon 171 is 5.4 cgm., and in the case of capon 148 is 5 cgm.

4. Capon 171 requires $20 \div 5.4 = 3.7 \text{ cgm.}$ of pulp to produce 1 cgm. of feather, and capon 148 requires $18.5 \div 5 = 3.7 \text{ cgm.}$ to produce 1 cgm. of feather.

B. BREAST FEATHERS

The same method used for the saddle feathers was used also for the breast feathers to determine a curve of weights, as well as lengths of pulp and of feather during regeneration. However, the range of size of feather is so considerable in the breast tracts that, in using as many feathers as are necessary, a much higher degree of irregularity of the

curves than in the saddle tract was unavoidable. Accordingly, these figures were discarded and another method used.

Growth in length was measured *in vivo* in a White Leghorn cock for each of a group of 3 feathers as described on page 145; and a fourth feather from the same region, agreeing with the average of these three in age and measurements, was plucked and used for determination of weights and other purposes previously mentioned. The figures of weight were then multiplied ten times and are so entered in Tables 7 and 8. This

TABLE 8
WHITE LEGHORN COCK: POSTERIOR BREAST FEATHER*

Age (Days)	Total Weight (Cgm.)	Weight of Feather (Cgm.)	Weight of Pulp (Cgm.)	Percentage of Pulp Weight to Total Weight
13.....	27.5	13.5	14.0	50.9
14.....	32.0	15.5	16.5	51.6
16.....	39.5	20.0	19.5	49.4
18.....	45.7	24.5	21.2	46.4
20.....	52.5	28.5	24.0	45.7
22.....	60.0	32.5	27.5	45.8
24.....	65.0	36.0	29.0	44.6
26.....	69.5	40.0	29.5	42.4
28.....	73.5	43.0	30.5	41.5
30.....	77.0	46.5	30.5	39.6
32.....	79.5	50.0	29.5	37.1
34.....	81.5	53.0	28.5	35.0
36.....	82.5	55.0	27.5	33.3
38.....	83.2	57.5	25.7	30.9
40.....	83.5	59.7	23.8	28.5
42.....	83.5	61.7	21.8	26.1
44.....	83.5	63.5	20.0	24.0
46.....	83.2	65.0	18.2	21.9
48.....	83.0	66.0	17.0	20.5
50.....	82.5	67.5	15.0	18.2
52.....	81.3	68.5	12.8	15.7
54.....	79.7	69.5	10.2	12.8
56.....	77.2	69.0	8.2	10.6
58.....	73.5	68.0	5.5	7.5
60.....	70.0	66.5	3.5	5.0
62.....	65.0	65.0	0.0	0.0

* Weight of pulp in relation to total weight of feather recorded for a single feather; each entry selected to correspond as closely as possible to the feathers measured for length in Table 2.

method has the disadvantage of using only a single feather for each determination; but, on the other hand, the advantage of control by known feathers.

This was done separately in the anterior part and the posterior part of the breast tract, and the two independent results are a check on each other. The measurements are given in Tables 7 and 8, and the data of Table 8 are entered in graphs (Fig. 17).

The same calculations may now be made as in the case of the saddle feathers:

Anterior breast.—

1. The average weight of 1 mm. of pulp during the entire period of regeneration is $30.74 \div 215.2 = 0.143$ cgm.

2. The total weight of pulp produced for each feather during regeneration is $79 \times 0.143 = 11.297$ cgm.
3. The total weight of 1 feather produced during regeneration is 4.1 cgm.

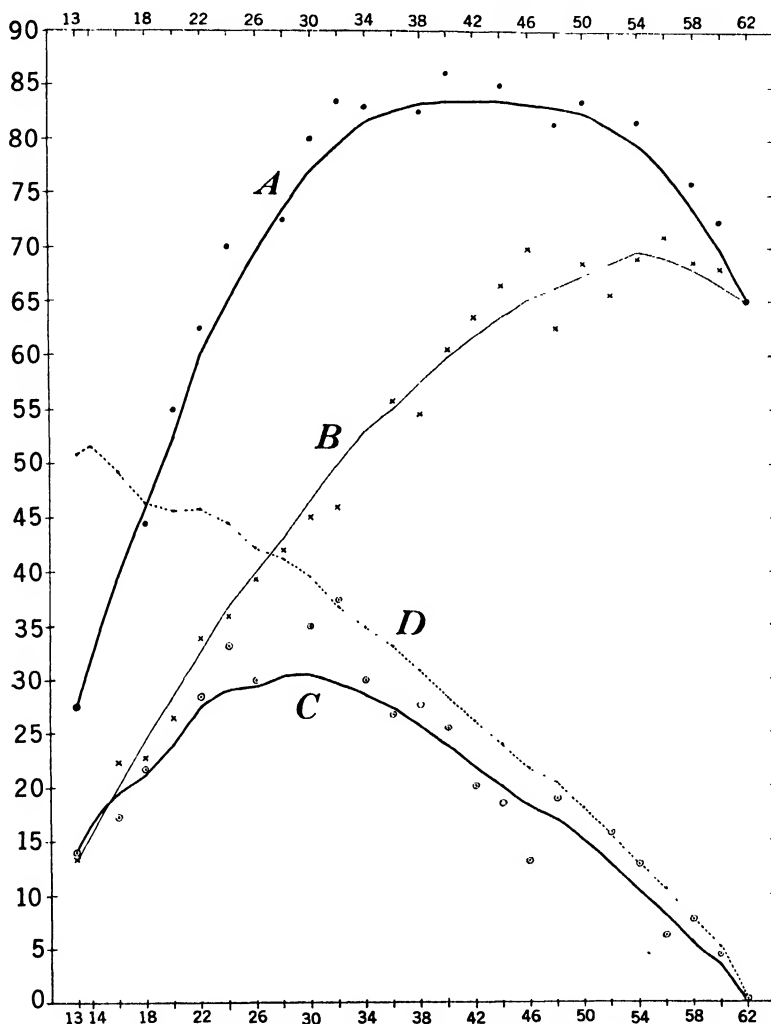


FIG. 17.—Curve of weights of constituents of regenerating posterior breast feathers, White Leghorn cock. Data from Table 8. *A* = total weight; *B* = weight of feather; *C* = weight of pulp; *D* = percentage of weight of pulp to total weight. Abscissae = days of regeneration; ordinates = centigrams for curves *A*, *B*, and *C*, percentages for curve *D*. Weight is ten times the weight of a single feather.

4. Pulp weighing 11.3 cgm. has produced 4.1 cgm. of feather, a ratio of 2.76:1.¹²

In order to gain an idea of the quantity of pulp required for a complete molt, let us

¹² In order to provide an independent check on the ratio between weights of pulp and feather, 108 regenerating feathers from the breast of a Brown Leghorn were plucked on the eighteenth and nineteenth

take a ratio of 3:1 as about the average ratio of weights of pulp and feathers.¹³ All the feathers of a young White Leghorn cock, weighing 1,642 gm., were found to weigh, after drying, 105 gm.; the pulp requirement for a complete molt in the case of this bird would thus be 315 gm. The dry weight of the feathers plus pulp, 420 gm., is 25.5 per cent of the weight of the entire bird. This gives a rough idea of the quantitative strain of molting, quite apart from the specific chemical requirements of the metabolism involved. According to our actual determination, the pulp contains 93.65 per cent of water.

IV. THE BLOOD VESSELS OF THE PULP¹⁴

I. INTRODUCTION

The blood vessels of the pulp were studied by injection with gelatin carmine for the most part; but for confirmation of certain points, sections of uninjected feather germs were also used. We have not yet succeeded in studying the circulation in the pulp *in vivo*. Areas with regenerating feathers of known, or in some cases unknown, age were selected; the skin was reflected with the bird under anesthesia, and a small artery, or in some cases a vein, was injected peripherally through a fine hypodermic needle with the warmed mass. In this way a considerable number of feather pulps could be injected simultaneously. The mass sets rapidly. The injected area was then excised, hardened in formalin, and the feathers prepared individually for study.

After some experimenting with the Brown Leghorn, the White Leghorn was used exclusively in order to get rid of the obscuring effects of melanophores. The method of study most frequently used was to dissect the feather and follicle together from the skin and to bisect the pulp-containing portion with a sharp razor blade; both halves were then cleared and mounted together in balsam. Figures 18 and 19 were made from such preparations with aid from a series of stained longitudinal sections.

In other cases the follicle was opened and the injected pulp was dissected free from the sheath and barbs; the bottom of the follicle might be left attached, but reflected, to show relations of the blood vessels of the follicle¹⁵ to excurrent vessels of the pulp; other anatomical dissections were also made. Some entire pieces of injected skin, with the feathers cut off short, were cleared and mounted whole to show the relations between the external cutaneous vessels and the blood vessels of the pulp.

In such ways many preparations of breast, neck, and wing feathers of different stages of regeneration were made, of which over 150 have been retained as permanent preparations. The following account is based mostly on the breast feathers in order to simplify the matter as much as possible; but there seem to be no differences in principle between the feathers of the different tracts.

The blood circulation in the pulp is quite unique in certain of its features, owing to special functional requirements.

days, and the pulp and feather separated. The pulps were weighed in groups of five, and each feather was weighed separately. From these data the average weights per millimeter of pulp and feather were calculated to be 0.172 cgm. and 0.0516 cgm., respectively—a ratio of 3.33:1.0.

¹³ No account has been taken in these calculations of the amount of the sheath lost during regeneration.

¹⁴ There are no previous accounts of the circulation in the pulp. Peska (1927) described the first origin of the vascular loop in the embryonic feather papilla.

¹⁵ It may be noted incidentally that the walls of the follicle have an extremely rich and characteristically arranged blood supply.

1. Growth of the feather is so rapid that a very rich blood supply is necessary, especially in the basal region adjacent to the collar, where all the plasmatic growth occurs; an especially rich capillary network occurs here.

2. The need for constant resorption and removal of the apex of the pulp at the same rate that it grows basally requires a very extensive apical capillary network; the pulp membrane and apical membrane also need nutrition for their extensive and rapid periods of reconstitution. Hence the maintenance of a large arterial supply and excurrent system to and from the apex of the pulp.

3. Another important requisite to be fulfilled in the blood system of the pulp depends on the fact that it is constantly growing out with the pulp at a rate of 2-3 mm. per day or even faster. It follows that the vascular system, like the epidermal system and the pulp itself, is constantly regenerating at its base at the same rate as they. Hence, in order to preserve a rather constant pattern throughout the greater part of the period of regeneration, as it does, it is necessary that it be extremely labile. Only the arteries possess adventitious coats, and the entire system within the pulp, whether in the form of capillaries or large sinuses, is otherwise exclusively endothelial. It is only within the papilla that the excurrent vessels acquire adventitious coats and may properly be denominated "veins."

4. The relatively narrow portals of entry and exit of the blood (Figs. 18 and 19) suggest a mechanism for retention of a large amount of blood constantly within the pulp.

2. THE MORPHOLOGY OF THE VASCULAR SYSTEM OF THE PULP

After these preliminaries we may proceed next to describe the actual vascular pattern of a given stage of the breast feather. Each regenerating feather is supplied by a single axial artery, derived from a cutaneous artery (Fig. 18), which traverses the entire length of the pulp and ends in an apical arborization. The part of the axial artery within the papilla is very thick walled (cf. Pl. II, Fig. 1). Its lumen through the papilla is extremely narrow in the preparations, although usually with a single knotlike swelling. At the apex of the papilla the lumen enlarges suddenly into the axial artery of the pulp, which is relatively very thin walled, although it and the main branches possess adventitious coats.

The branches of the axial artery as shown in Figure 18 are based on a single preparation. It will be noted that those at the base are relatively small, which, at first sight, appears incongruous in view of the vascular needs of the corresponding portion of the feather germ; but, as explained before, the entire system is moving out with the growth of the pulp, and the branches toward the base are the newer ones.

Photographs of the apical arborization of the axial artery are shown in Plate IV, Figures 22 and 23. Figure 22 represents the arborization at about stage II of the resorption of the pulp, and Figure 23 at about stage IV.

The blood in the branches of the axial artery passes into capillaries, which reunite to form the excurrent system, as shown in Figure 19, the pattern of which is likewise based on a single preparation. All the capillaries and excurrent vessels have only endothelial walls, no adventitious coats whatever being present.

The outstanding features of the excurrent system¹⁶ (Fig. 19) are: (1) The series of large pericentral sinuses which occur invariably in the basal portion of the pulp and

¹⁶ As the excurrent vessels within the pulp are entirely devoid of adventitious coats, these vessels are not denominated "veins."

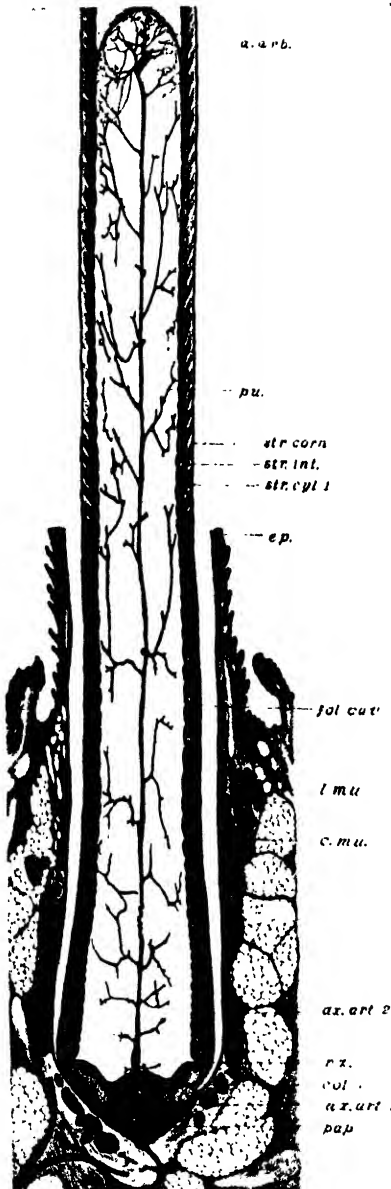


FIG. 18

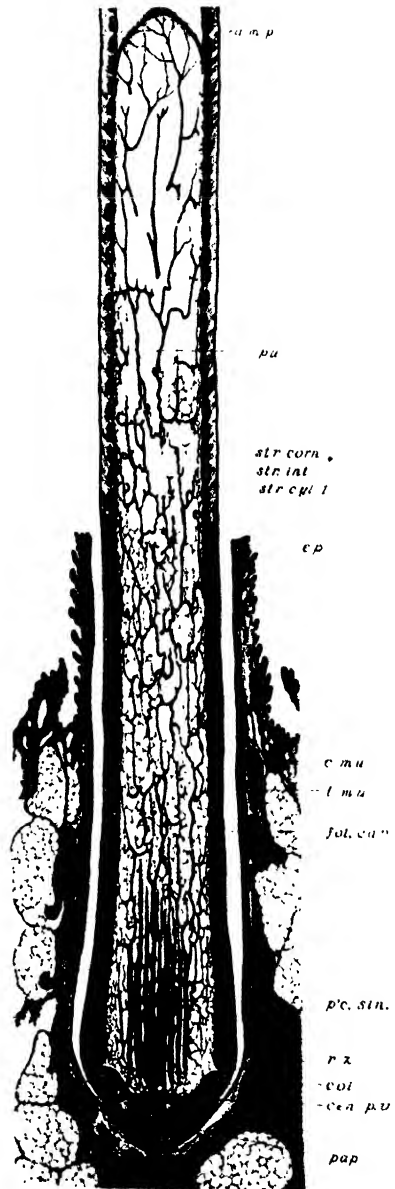


FIG. 19

FIG. 18.—Semi-diagrammatic representation of the arterial circulation in the pulp and papilla of a breast feather on the twenty-first day of regeneration, White Leghorn cock. $\times 18$. By Hsi Wang. See table of abbreviations, p. 175.

FIG. 19.—Semi-diagrammatic representation of the capillary and excurrent system of the pulp and papilla of a breast feather on the twenty-first day of regeneration, White Leghorn cock. $\times 18$. By Hsi Wang. See table of abbreviations, p. 175.

occupy about one-seventh of its entire length. The photographs shown in Plate II, Figure 1, and Plate I, Figure 4 (cf. also Pl. IV, Figs. 20 and 21), give an excellent idea of the disposition of the sinuses. (2) The extremely narrow true veins within the papilla arising from the looped connections of the pericentral sinuses. These veins are irregular in number; some of them occupy the central region of the papilla and hence are called "central papillary veins"; others, "peripheral papillary veins," are found on the periphery of the papilla. It will be seen (text Fig. 19 and Pl. IV, Fig. 21) that they communicate externally with sinuous cutaneous veins. (3) The very richly developed capillary network at the periphery of the pulp in the region occupied by the sinuses, which we regard as related to the very active growth in this region.

Figures 20 and 21 of Plate IV are photographs of longitudinally bisected feather germs, including the region of the papilla and part of the region of the sinuses.

3. DISCUSSION

The general morphology of the system is related, as suggested in the introduction to this section, to the functional requirements of metabolism, resorption, and maintenance of a constant rich supply of blood in the pulp. In considering these relations, we should discuss the course of the blood stream, the pattern in different stages, the lability and regulation of the pattern with reference to the continuous outward growth of the pulp, and the special features of pattern in different tracts.

As stated before, we have not succeeded in observing the course of the blood stream within the pulp *in vivo*. Dr. Melvin Krisely was so kind as to aid us in special illumination of the pulp-containing part of the growing feathers of young White Leghorn chicks, but it was found to be impossible to obtain any clear views through the sheath and ridges of the germ. Exposure of the pulp in such a way as to preserve normal circulation was also unsuccessful.

We have to depend, therefore, upon the anatomical relations exclusively for conclusions concerning the course of the blood within the pulp. The general course is unmistakable. The axial artery and its branches distribute the blood at various levels, in the manner shown in Figure 18, to the capillary network which is predominantly peripheral. These capillaries reunite in larger, more central vessels, which, like the capillaries, have only endothelial walls; and these lead into the large basal pericentral sinuses, also with exclusively endothelial walls, which discharge through the central and peripheral papillary veins in the manner already described.

The axial artery and the veins within the papilla possess very narrow lumens relative to their connections within the pulp. It would be expected that the process of fixation necessary to the preservation of specimens for study would tend to contract the walls, and hence it is not safe to conclude that the condition found in fixed specimens is typical of their condition under all functional conditions. The walls of the axial artery are very thick (cf. Pl. II, Fig. 1); the appearance of an enlargement of the lumen at the base of the papilla, as shown in Figure 18, is very common. It is quite probable that the lumen of the artery throughout its extent is capable of dilation and contraction, and thus of regulating the amount of blood admitted to the pulp.

The capacity of the papillary veins collectively is greater than that of the single axial artery. In some preparations they appear greatly distended. Possibly the muscular substance of the papilla controls this to some extent.

It is not certain that all of the blood passes through the sinuses or that all of the

blood in the sinuses is on the direct way out. The cross-connections between the sinuses and the peripheral capillaries may be two-way channels responsible in part for the metabolic requirements of this very active portion of the feather germ; the arterial system is, in fact, very poorly developed in this region, owing to its constant renewal as the pulp grows out from the papilla.

The narrowness of the portals of entry and exit of blood with relation to the capacity of the vessels of the pulp can best be interpreted as a device to store and conserve within the pulp quantities of blood adequate for the great demands of growth and resorption. It is quite probable that the substance of the papilla acts as a regulator of the amount of blood within the pulp. The sinuses might then be interpreted as reservoirs to meet the demands of the growing region of the germ so that this would not be subject to sudden fluctuations. The quantitative demands for blood during replacements after molting or extensive plucking are great and undoubtedly also involve changes in the general cutaneous blood system; but the highly localized requirements of the individual feather can best be met by local adaptations.

We have also considered the question whether the wall of the follicle might function by contraction in regulating the amount of blood in the pulp. The strong layer of circular muscles in the wall next to the lining epidermis (Figs. 18 and 19) suggests this possibility. Experiments by Kuhn (1932) and Woitkewitsch (1934a, 1934b) agree in showing that the follicle plays no necessary role in regeneration of the feather. We have also confirmed this conclusion. The follicle may be opened from top to bottom, so as to prevent any compression by it on the growing feather without hindering regeneration. Woitkewitsch even diverted the growing feather outside its follicle through a small opening made at its base, with the result of a mere retardation of rate of growth as consequence. We conclude, therefore, that the wall of the follicle cannot play an important role in regulating the blood supply within the pulp.

A final suggestion on regulation of the blood supply within the pulp concerns the question of turgor within the pulp itself. Although the pericentral sinuses may be full of blood, practically none of it escapes when the regenerating feather is plucked. But if the base of a freshly plucked regenerating feather is gently pressed, a clear serum¹⁷ exudes, along with a smaller amount of blood. The question is thus suggested whether turgor of the pulp is maintained by the serum and may be a factor in regulating the amount of blood within the pulp.

That turgor of the pulp is not sustained by direct blood pressure appears to be proved by the fact that even considerable loss of blood after amputation of apical fractions of the pulp causes only a brief halting of the regeneration of the feather (unpublished observations). The plain inference of this fact is that the relation between the pulp and the ectodermal cylinder has not been seriously disturbed, and that shrinkage of the pulp due to loss of blood does not occur. There is thus only an indirect relation between the blood and the turgor of the pulp. But the relationship is, nevertheless, very immediate, for if the blood supply of the regenerating feather is cut off by ligaturing the axial artery, the pulp does not swell when the sheath is removed, as it does immediately if the circulation be left intact (see p. 144); this demonstrates that turgor of the pulp is lost very rapidly after the circulation is shut off, though persisting after bleeding

¹⁷ A similar clear serum also exudes from the tip of the pulp when the apical membrane is removed. It is evident that, in addition to extracellular supporting substance, there is a fluid extravascular component of the pulp.

at the apex. The indications are that the pulp is constantly extracting fluid from the blood stream and that it is this fluid which is responsible directly for the turgor of the pulp.

The functional requirements of the circulation at different stages are essentially the same up to the time when the definitive diminution of the pulp begins (Figs. 2 and 3). Correspondingly, the general pattern of vascularization is the same throughout the entire plateau period of length of the pulp. This is the case in spite of the fact that the entire system is constantly being carried out by the basal growth of the pulp and new vascular elements are being formed in the new growth. Even at 9 days of regeneration in the breast feathers of White Leghorns, one finds the same narrow portals of entry and exit, the axial artery with its apical arborization, and the large basal pericentral sinuses. During the period of definitive diminution of the pulp, as shown in Figures 2 and 3, the principal change appears to be in the reduction of the pericentral sinuses.

The constancy of general pattern with reference to fixed levels from the base of the germ, in spite of continuous outward growth with reference to these levels, demonstrates a high degree of capacity for regulation of the system. This is based on the fact that, with the exception of the arterial system, the entire complex is endothelial and is embedded in a loose and pliable mesenchyme. The system is therefore free to expand or to contract with reference to the pressure and volume of the blood flow. This, in its turn, bears an obvious relation to the state of functional activity at various levels, which must be supposed to control vascular reactions according to well-known biological principles.

The pulp, growing constantly from the surface of the papilla, is supplied with capillaries as rapidly as it is formed, and this does not seem to involve any adaptations special to the particular system. It is different, however, in the case of the axial artery, which requires adaptations special to the moving system of which it forms a part. In the first place, its base must be continuously renewed without detriment to the circulation; and, in the second place, the new portion of the pulp must be supplied from time to time with new arteries.

The part of the axial artery immediately above the apex of the papilla exhibits special features correlated with these requirements. It is much dilated, as compared with the portion within the papilla; and there is a sudden transition from the thick wall of this portion to the much thinner wall within the pulp (Pl. II, Fig. 1). The dilation above the papilla is often considerably reduced apically, which would seem to indicate a readier yielding to the internal blood pressure at this place. Minute arteries bud out of the dilation. Although these buds end blindly in the pulp at first, they soon connect with the rich capillary system; and, as they are carried apically, they enlarge. Thus, at any given time the basal branches of the axial arteries are the smallest ones, and there is a progressive increase in size apically, for a certain distance at least (see Fig. 18).

The circulation in the neck hackles and in the wing primaries has also been studied in numerous injected specimens for comparison with breast feathers; other tracts were not studied. The general pattern of the circulation is the same as in the breast feathers. Of course, numerous differences in detail appear. In the wing primaries there is a great increase of scale in diameter and especially in length, with a corresponding increase in the capacity of the main vessels. The sinuses appear to be more numerous, and the central veins of the papilla decidedly larger, whether relatively so or not. But it is clear

that there is a type of circulation in the pulp of regenerating feathers to which all those studied conform.

V. SUMMARY

The growth of the pulp of regenerating breast and saddle feathers of White and Brown Leghorn fowl has been examined with reference to the growth of the feather proper. The pulp grows in length at the same rate and to the same extent as the feather. However, after about the fifteenth day of regeneration periodic resorption of the apex of the pulp equals the growth, which proceeds from the base, so that a certain standard, or functional, length of pulp is maintained throughout the greater part of the period of regeneration until about the beginning of formation of the calamus, after which growth slackens and the pulp is finally completely resorbed in periodic steps marked by the formation of the scala of the calamus.

The periods of resorption of the pulp of about 12 hours' duration are approximately daily, though not conforming to any fixed hours of day or night. Periods of reconstitution of about equal duration alternate with them. The periods of resorption are marked by a profuse phagocytosis in the pulp and by splitting of the thickened ectodermal apical membrane of the pulp to form the cavity of a pulp cap. During reconstitution the apex of the pulp and the apical membrane are restored. A single keratinized, thimble-shaped pulp cap is formed in each period of resorption from the apical membrane. During earlier stages of regeneration, although the pulp is steadily increasing in length, periodic resorption begins on the eighth day; but, as long as the apex of the sheath is intact (pinfeather stages), a series of delicate partitions only is formed across the apical cavity between the tip of the pulp and the closed apex of the sheath. These differ from the typical pulp caps and resemble in miniature the scala of the calamus.

The weight of pulp and of feather was determined at 2-day intervals from the thirteenth day of regeneration. From these figures it was calculated that the ratio of weight of pulp to that of feather produced is of the order of 3 to 1, and that the weight of pulp and feather required for a complete molt is of the order of 25 per cent of the total weight of the bird.

The circulation in the pulp is described for the first time, and remarkable adaptations for rapid growth of the feathers, periodic resorption of pulp, and maintenance of a constant pattern in the moving substratum of the pulp are discussed.

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ABBREVIATIONS USED IN ILLUSTRATION LEGENDS

- | | |
|---|--|
| <i>a. arb.</i> = apical arborization | <i>m. a. m.</i> = middle layer of the apical membrane of the pulp |
| <i>a. c.</i> = apical cavity | <i>m. l. a. m.</i> = Malpighian layer of the apical membrane of the pulp |
| <i>a. m. p.</i> = apical membrane of the pulp | <i>pap.</i> = papilla |
| <i>ax. art. 1</i> = axial artery in the papilla | <i>p'c. sin.</i> = pericentral sinuses |
| <i>ax. art. 2</i> = axial artery in the pulp | <i>per. p. v.</i> = peripheral papillary veins |
| <i>ax. pl.</i> = axial plate | <i>p. m.</i> = pulp membrane |
| <i>b.</i> = barb; section of column of cells that will form the barb | <i>pu.</i> = pulp |
| <i>b'bl. pl.</i> = barbule plate | <i>pu. c.</i> = pulp cap |
| <i>b. m.</i> = barb membrane | <i>r.</i> = ridges |
| <i>cen. p. v.</i> = central papillary veins | <i>rh.</i> = rhachis |
| <i>c. mu.</i> = circular muscles of the wall of the follicle | <i>r. z.</i> = ramogenous zone |
| <i>c. p. c.</i> = cavity of pulp cap | <i>sh.</i> = sheath |
| <i>col.</i> = collar | <i>str. corn.</i> = stratum corneum (forms sheath) |
| <i>d.</i> = follicular debris | <i>str. cyl. 1</i> = stratum cylindricum: the part in contact with the pulp destined to form the pulp membrane and the barb membrane |
| <i>ep.</i> = epidermis | <i>str. cyl. 2</i> = stratum cylindricum: the adjacent layers which form a barb septum |
| <i>fol.</i> = wall of follicle | <i>str. int.</i> = stratum intermedium (forms feather) |
| <i>fol. cav.</i> = cavity of follicle | <i>v.</i> = ventral |
| <i>k. a. m.</i> = keratinized layer of apical membrane | |
| <i>l.</i> = basal reuniting loops of the pericentral sinuses from which central papillary veins arise | |
| <i>l. mu.</i> = levator muscle of the feather | |

PLATE I

FIG. 4.—Transverse section, breast of White Leghorn cock; 19 days' regeneration. $\times 43$. The level of the section is a short distance above the papilla (cf. Pl. II, Fig. 1; text Fig. 19; and Pl. IV, Fig. 20).

FIG. 5.—Part of Fig. 4. $\times 533$. N.B.: The cells of the ridge bounded by the stratum cylindricum and the stratum corneum (sheath) constitute collectively the stratum intermedium.

PLATE II

FIG. 1.—Frontal longitudinal section through the base of a regenerating breast feather, White Leghorn cock; 19 days' regeneration. $\times 36$.

FIG. 8.—Photograph of longitudinal section of stage I of pulp-cap formation, breast feather, Brown Leghorn capon; 25 days' regeneration. $\times 36$. (Cf. text Fig. 7.)

FIG. 9.—Photograph of longitudinal section of stage II of pulp-cap formation, breast feather, Brown Leghorn capon; 25 days' regeneration. $\times 36$. (Cf. text Fig. 7.)

FIG. 10.—Photograph of longitudinal section of stage IV of pulp-cap formation, breast feather, Brown Leghorn capon; 25 days' regeneration; keratinization, beginning in *m. a. m.*, does not correspond exactly to diagram, text Fig. 7, IV. $\times 36$. (Cf. Fig. 7.)

PLATE III

FIG. 11.—Photograph of pulp caps showing incomplete resorption; from regenerating breast feathers, Brown Leghorn capon. $\times ca. 7$.

FIG. 12.—Dried pulp caps showing barb septa in position. *A*=dorsal view showing rhachis depression in center; *B*=lateral view; *C*=another dorsal view. *A* and *B*=Brown Leghorn capon, breast, 27 days' regeneration; *C*=Brown Leghorn capon, saddle, 25 days' regeneration. $\times 6$.

FIG. 13.—Dried pulp caps after loss of barb septa, breast feather, Brown Leghorn capon; 25 days' regeneration. *A*=three caps held together by barb membrane; *B*=two caps breaking apart. $\times 6$.

FIG. 14.—Regenerating saddle feathers, Brown Leghorn capon; 33 days' regeneration, with persistent sheath and all pulp caps in place. $\times ca. 2$.

PLATE IV

FIG. 20.—Photograph of basal portion of a bisected injected feather germ to show the pericentral sinuses and capillaries, breast feather, White Leghorn cock; 21 days' regeneration. $\times 44$.

FIG. 21.—Photograph of basal portion of a bisected injected feather germ to show the pericentral sinuses and capillaries and one of the central papillary veins; breast feather, White Leghorn cock; 21 days' regeneration. $\times 44$.

FIG. 22.—Apical arborization of regenerating breast feather of a juvenile White Leghorn, with complete pulp cap above it; stage II of resorption; dissection of injected specimen. $\times 44$.

FIG. 23.—Apical arborization of breast feather, White Leghorn cock; 21 days' regeneration; stage IV of resorption as shown by structure of apical membrane; bisected injection. $\times 50$.

PLATE I

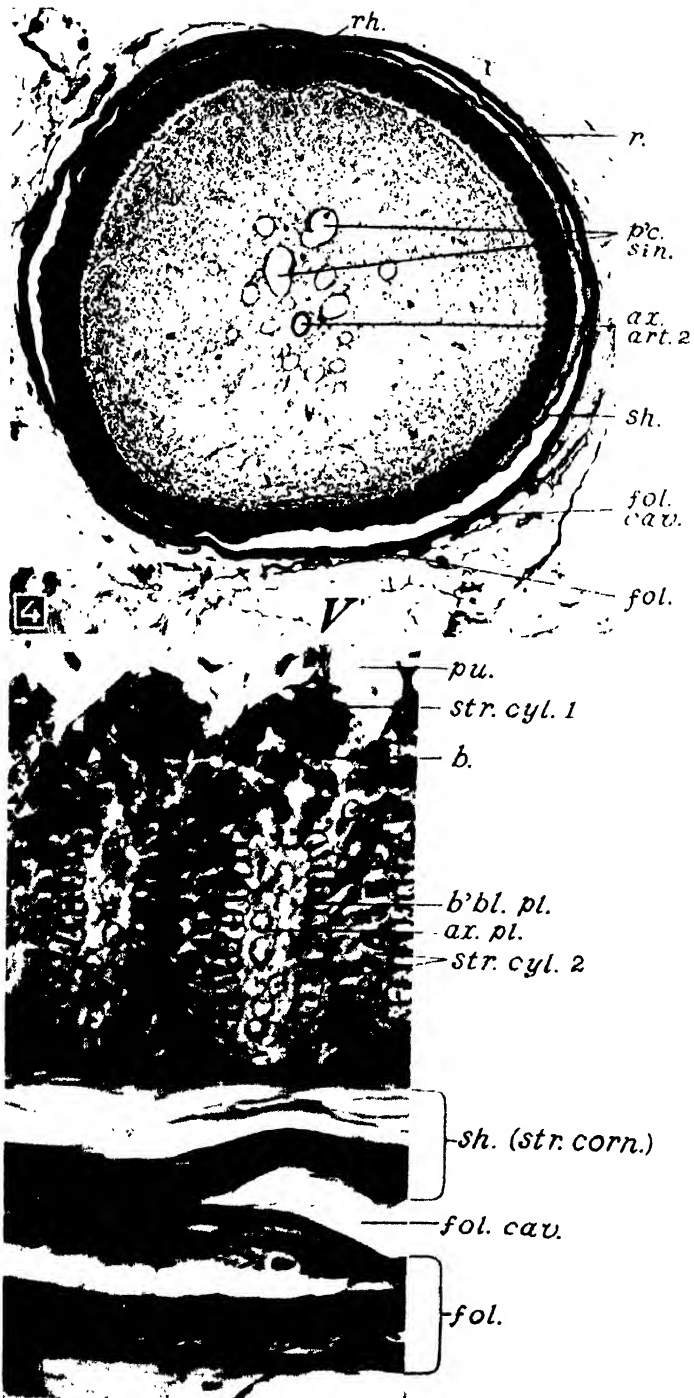
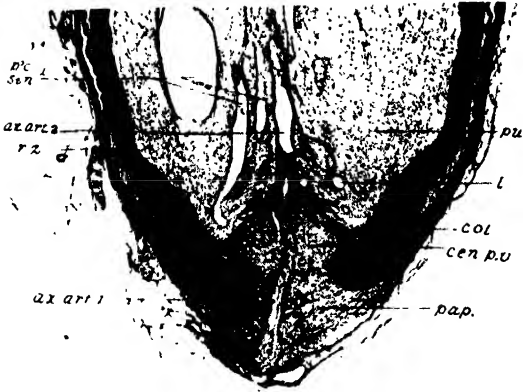
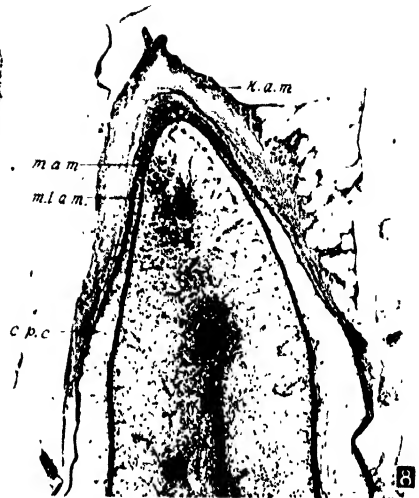


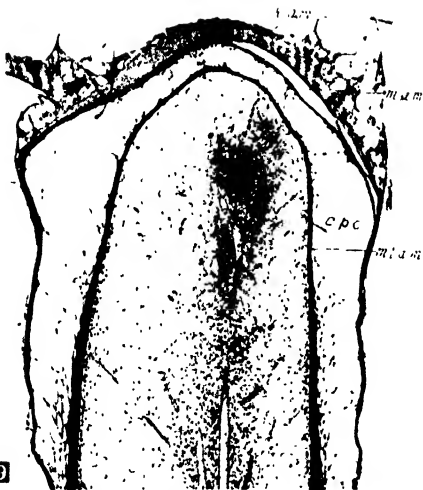
PLATE II



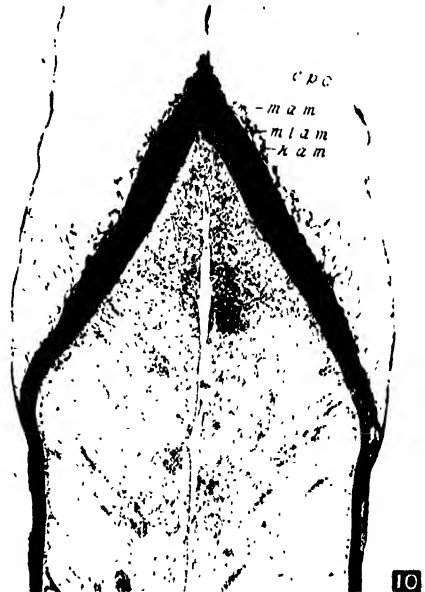
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4

PLATE III



11



14



12



PLATE IV



THE CONTROL OF FEATHER COLOR PATTERN BY MELANOPHORES GRAFTED FROM ONE EMBRYO TO ANOTHER OF A DIFFERENT BREED OF FOWL

(One text figure; seven plates)

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THE present study had its origin in experiments designed to analyze the behavior of skin transplants made between chick embryos of genetically different breeds of fowl (Willier, Rawles, and Hadorn, 1937). By grafting a piece of skin ectoderm from the head to the wing—regions differing greatly in the arrangement, size, and shape of feathers—it was hoped that some light might be thrown upon the problem of the distribution of feather germs (i.e., their arrangement and spacing in tracts) and the localization and nature of the factors controlling their rate of growth, shape of feather produced, etc. The effects of the mesodermal substratum and humoral agents of the host on such developmental processes could thus be tested. In grafting the skin of one embryo to another of a different breed, a means is furnished for analyzing the expression of the different genetically determined potentialities of the combined tissues in the characterization of the feather.

When such host-graft combinations are made, an area of donor-colored down feathers is formed at and about the site of grafting in the great majority of cases. The down of these areas is replaced, after hatching, by juvenile contour feathers having the shape, rate of growth, and distribution in tracts characteristic of feathers occurring in corresponding regions of host-control chicks but invariably with the color or color pattern of the donor breed.

In seeking a solution to the problem of the manner of origin of the area of donor-colored feathers, it was found that this area did not arise solely by growth and spread of the implanted piece. It has been proved that the feathers of this area are derived structurally from host epidermis. The color, however, is produced under the control of melanophores derived from the donor tissue. Histological studies show that the implanted skin ectoderm, although incorporated at the implantation site, replaces little, if any, of the host epidermis of the affected area (Willier and Rawles, 1938*a*, 1938*b*, 1938*c*). Apparently at the time of implantation the skin ectoderm (from head region) was already "infected" with melanoblasts which had wandered in from the neural crest. From evidence now available, it is clear that these pigment cells migrate out from the implant into the host epidermis and the feather germs developing from it and produce the area of donor-colored feathers.

The purpose of the present paper is to present a general survey of the results obtained by grafting skin ectoderm and mesoderm from the embryo of one breed of fowl to another. In the analysis of the results, consideration will be given to several outstanding topics: (1) the respective roles that the donor and host play in feather characterization within the area of donor coloration; (2) the source and migration of the donor melanophores; (3) the breed-specific control of feather color or color pattern by the

donor melanophores, and the extent to which this control is independent of factors in the host; and (4) the peculiar constitution and behavior of the White Leghorn melanophores.¹

BREEDS AND METHOD OF GRAFTING

For this investigation graft-host combinations between embryos of the following breeds were tested: Barred Plymouth Rock, Black Minorca, Buff Minorca, F₁ hybrid (from Barred Plymouth Rock ♀ × Rhode Island Red ♂), New Hampshire Red, White Silkie bantam, White Wyandotte, White Plymouth Rock, and Single Comb White Leghorn. For types of combinations tested see Table 1.

The method employed is that of grafting a piece of skin ectoderm or pure mesoderm from an embryo of one breed to another of the same age. The piece of skin ectoderm used is small, measuring 0.5–1.0 × 0.5 mm. The region from which the skin ectoderm is taken is first stained with Nile blue sulphate, so that the isolate can be easily seen during the subsequent handling and grafting. It is stripped from the body of the embryo by means of fine steel needles. During the isolation the embryo and pieces removed are kept until grafted in Locke's physiological salt solution, warmed to a temperature of 38° C. on a thermostatically controlled stage-warmer.

The source of skin ectoderm was usually the dorsolateral surface of the head anterior to the otocyst; but in a few cases it was taken from the wing bud, leg bud, or dorsal surface of a somite of the trunk region. To such pieces a small amount of mesenchyme always adheres. A histological study of pieces isolated from the head, however, shows that the number of mesenchyme cells is very small. When pure mesoderm was used for implantation, it was usually isolated from the limb buds.

The host embryo is prepared for receiving the implant by first sawing a window in the shell directly over the embryo, whose position is determined by candling. The shell over the window is removed, and enough of the shell membrane to expose the body of the embryo. The embryo is now viewed through a binocular dissecting microscope, and the site for transplantation located. This is usually the base of the wing bud; but in a few cases the graft was made to the dorsal surface of the head, to the leg bud, and to the tail bud. With very fine steel needles an opening is then made in the chorion and amnion over the selected site.² Through these openings an incision is now made with a glass needle in the skin ectoderm at the base of the wing bud, leg bud, or other locus selected for implantation. See Plate I, Figure 2. The isolated piece is carefully pushed into the incision—deep enough into the host mesoderm to anchor it in position. Often the free end is more or less in contact with the surrounding host ectoderm. The piece of shell is now returned to the window, sealed in position with melted paraffin, and the egg returned to the incubator.

The egg, during and subsequent to the operation, is kept on a nest of cotton on a Syracuse watch glass with the window surface uppermost. The majority of chicks hatch normally, but some have considerable difficulty in emerging from the shell and have to be helped out by carefully picking away the shell at the large end of the egg.

¹ We take this opportunity to express to Professor H. B. Hutt and Dr. J. H. Bruckner, of Cornell University, our appreciation of their friendly interest in this work and their co-operation in furnishing us with eggs from a cross between Rhode Island Red and Barred Plymouth Rock breeds.

² These membranes are usually stained with Nile blue sulphate to make them more easily seen. The staining is carried out very conveniently by means of an agar-coated glass rod dipped in a solution of the dye.

GENERAL STATEMENT OF RESULTS

The data obtained by grafting a piece of head skin ectoderm from embryos of one breed to the wing bud of another are summarized in Table 1. From an inspection of this table it will be seen, in the first place, that all possible types of combinations have been made between donor and host embryos of different breeds having either pigmented or white feathers. These are: (1) pigmented to white, (2) pigmented to pigmented, (3) white to pigmented, and (4) white to white-feathered breeds.

Secondly, skin ectoderm from embryos of breeds with pigmented feathers or with white feathers grafted to embryos of breeds having either white or pigmented plumage results in the formation of an area of donor-colored feathers on the wing and adjacent regions (see Figs. 4-10). Lastly, it will be noted that host-graft combinations of the various white-feathered breeds (Leghorn, Plymouth Rock, and Wyandotte) produce white, not pigmented, feathers. The donor-colored area, if produced, is indistinguishable from the host-colored feathers.

The table also shows whether or not an area of donor-colored feathers is produced in the host, and the frequency of occurrence. This is indicated for both the embryos which failed to hatch and the chicks which hatched.

In another, less extensive series of experiments with the same breed combinations, both the source and the site of transplantation were varied. Pieces of skin ectoderm and of pure mesoderm from the head, trunk (somite level), wing buds, and leg buds were grafted to the head, tail, and leg-bud regions of host embryos. At all ages tested (62-108 hours), donor skin ectoderm or mesoderm from the head or the trunk region produced an area of donor-colored feathers irrespective of where the transplant was placed. The results were briefly as follows: head skin ectoderm to tail bud, 14 positive, 6 negative; to leg bud, 2 positive, 1 negative. Skin ectoderm from the trunk region transplanted to the head, 2 positive, 1 negative; to the wing, 10 positive, 10 negative. When, however, the transplant was taken from the wing or leg buds, the age of the donor was of great importance in determining whether a positive or a negative result would be obtained. For example, wing skin ectoderm taken from donors 62-79 hours and transplanted to the head of a host embryo gave 17 negative and no positive results; but when the donors were 80-103 hours old, 9 negative to 13 positive cases were obtained. Similarly, wing skin ectoderm from donors 70-80 hours grafted to the wing region gave 11 negative and no positive cases, as compared with 3 negative and 7 positive cases obtained when the donors were 80-132 hours old. Also, leg-bud skin ectoderm from donors 94-103 hours gave 10 negative and no positive cases when transplanted to either the head or the wing-bud regions, while no negative and 2 positive cases resulted when the donor was 132 hours old.

Grafts of pure mesoderm gave results, in general, like those just described for skin ectoderm. Head mesoderm (72-100 hours) grafted to the wing gave 13 positive to 2 negative cases; mesoderm from the wing bud of donors 96-116 hours grafted to the host wing bud produced 93 positive and 95 negative cases (see Willier and Rawles, 1938c, Fig. 2).

Lastly, an examination of the results from all the various types of experiments in which the White Leghorn served as donor shows that it does not often produce an area of white feathers. So far, no positive results have been obtained with F₁ hybrid and New Hampshire Red host embryos, and very rarely with Barred Plymouth Rock hosts. Table 1 shows that, out of 18 cases in which White Leghorn head skin ectoderm was

TABLE 1
SUMMARY OF DATA ON GRAFTING HEAD SKIN ECTODERM TO WING BUD

DONOR		HOST						
Breed	Age (Hours)	Breed	Age (Hours)	Em- bryo Dead before Feath- ering	Area of Donor-colored Feathers			
					No. of Positive Cases		No. of Negative Cases	
					Embryo*	Hatched	Embryo*	Hatched
Black Minorca	68-76; 108	White Leghorn	68-76	7	8	13	3	5
Black Minorca	72, 114	White Wyandotte	72, 91	0	1	0	4	5
Buff Minorca	70-76	White Leghorn	68-75	3	13	6	5	2
Buff Minorca	73, 77	White Wyandotte	73, 77	2	1	2	0	1
Barred P. Rock	71-78; 82, 106	White Leghorn	71-84	17	26	15	7	9
Barred P. Rock	74, 77	White Silkie	74, 77	14	5	0	7	0
Barred P. Rock	76, 80, 98	White P. Rock	72-73	0	2	2	3	1
Barred P. Rock	73-76; 80	White Wyandotte	73-76	7	5	4	4	4
N.H. Red	75-76; 83	White Leghorn	72-76; 83	1	7	4	1	1
F ₁ hybrid	63-101	White Leghorn	65-100	36 (9)†	69 (9)†	45	16 (3)†	16
Black Minorca	72-76	Barred P. Rock	70-76	1	0	4	11‡	10
Barred P. Rock	77	Black Minorca	99	1	0	0	1‡	1§
Barred P. Rock	73-74	Buff Minorca	76-77	1	3	0	1	1
N.H. Red	71-79	Barred P. Rock	71-79	15	4	7	9	2
Barred P. Rock	71-82	N.H. Red	71-82	15	1	4	4	4
White Leghorn	71-75	Black Minorca	71-76	10	9	1	10	5
White Leghorn	71-76	Buff Minorca	72-76	2	1	1	6	4
White Leghorn	70-80; 91	Barred P. Rock	60; 70-84	9	2 (trace)	0	11	5
White Leghorn	72-76; 88-92	F ₁ hybrid	72-76; 88-92	(14)†	0	0	(11)†	0
White Leghorn	72-78; 83	N.H. Red	72-78; 83	12	0	0	14	13
White Wyandotte	74-76	Black Minorca	74-76	0	2	4	0	0
White Wyandotte	75	Buff Minorca	74	1	1	3	0	0
White Wyandotte	73-83	Barred P. Rock	73-80	12	8	8	4	2
White Wyandotte	72-73	F ₁ hybrid	72-78	2	0	3	2	2
White Silkie	70, 80-84	Black Minorca	70-75	10	11	3	1	0
White Silkie	70-80	Barred P. Rock	70-80	8	2	9	4	4
White Silkie	70-80	Barred P. Rock	70-80; 102-107	8	2	9	4	4
White Leghorn	82	White Wyandotte	73	0	0	0	1	1
White Leghorn	71-76; 80, 92	White P. Rock	71-76	2	0	0	8	12
White P. Rock	62, 63	White Leghorn	62, 63	3	0	0	6	3
White Wyandotte	70-79	White Leghorn	70-78	5	0	0	11	16

* Fully feathered; died before or at time of hatching.

† Numbers in parentheses are those killed and examined before hatching.

‡ Down feathers of host and donor indistinguishable.

§ Died before juvenile plumage emerged.

|| Down feathers often bluish gray, a characteristic also found in control White Plymouth Rock embryos.

transplanted to Barred Plymouth Rock, only 2 showed traces of white down on the right wing. Two other positive cases from many trials have been obtained with Barred Rocks—one from a transplant of skin ectoderm from the wing base of a $7\frac{1}{4}$ -day-old embryo and another from a transplant including the dorsal part of a somite and neural tube from the posterior region of a 78-hour embryo (see Fig. 11). With Black and Buff Minorca hosts the White Leghorn implant produced an area of white feathers on the wing with higher frequency, although never very extensive (head skin ectoderm to wing bud gave 12 positive to 25 negative; skin ectoderm overlying the somites of a 72-hour donor gave traces of white feathers in 3 out of 3 trials).

THE AREA OF DONOR-COLORED DOWN FEATHERS

The area of donor-colored down feathers has been produced in various regions of the body, depending upon the site of grafting. When grafted to the base of the right wing bud, donor-colored plumage develops usually on the entire surface of the wing (see Figs. 4-10). In some cases it covers either the humeral or the ulna-radial portions only. In still others the area is more extensive, covering not only the entire wing but adjacent parts of the breast, neck, rump, and thigh of the operated side. In these cases the donor-colored plumage does not often extend across the mid-ventral or mid-dorsal line (figs. 5 and 6). When the spread is greatest, a single area may include the humeral and alar tracts on the wing, the breast tract, a portion of the spinal tract, and the femoral and crural tracts of the leg. If the implant is pushed deeply into the body wall at the base of the wing bud, the donor-colored plumage may be confined to the breast. In general, the path of extension of the effect is toward the tip of the wing and ventrally to the mid-line of the breast.

The same donor effect may be produced on any region of the body irrespective of the source of the implant. If head skin ectoderm of a Barred Plymouth Rock or Buff Minorca embryo is placed into the tail bud of a White Leghorn embryo, the entire uropygium and adjacent regions of the back develop donor-colored feathers. Leg bud, wing bud, or back skin ectoderm (Barred Plymouth Rock, F₁ hybrid) grafted on the head of a White Leghorn produces a relatively small localized area of black down (Fig. 3). When head skin ectoderm is implanted to the base of the leg bud (F₁ hybrid to White Leghorn), an area of black down covers the thigh and shank of the leg and adjacent parts of the body.

In size and distribution the down feathers of the donor-colored area are identical with those occurring in corresponding regions of either the control embryos or the unoperated side (left) of the same embryo. Feathers on the wing have the form and arrangement characteristic of primaries, secondaries, and coverts.

A microscopical examination of the down feathers of the "graft" area shows that the barbs and barbules are entirely donor colored except those occurring at the margin. Here they are mosaics of donor- and host-colored barbs and barbules of variable proportions. The barbs and barbules are found to contain pigment granules of the donor variety when a pigmented breed is used, and no pigment granules when the donor is a white breed. The same observations have been made on the downy barbules of donor-colored feathers of the juvenile plumage and will be described more completely below.

REPLACEMENT OF THE DOWN WITH JUVENILE PLUMAGE IN THE
AREA OF DONOR COLORATION

After hatching, the donor-colored down feathers of the "graft" area are gradually replaced with contour feathers of the juvenile plumage. These have the color or color pattern of the donor breed (see Figs. 11-20). Skin ectoderm or limb-bud mesoderm of a Barred Plymouth Rock donor will bring about the formation of black and white barring pattern in the contour feathers of the wing, breast, etc., of a White Leghorn host. New Hampshire Red grafted to White Leghorn or Barred Plymouth Rock produces red feathers with black markings, as in the control. Feathers produced when F_1 -hybrid embryo is donor to White Leghorn host show sex-linked differences in color pattern. When the donor implants come from a male embryo, a barring pattern results; when from a female nonbarred feathers either solid black or with varying amounts of red pigment are formed (Fig. 20). These patterns developed on hosts of either sex and resemble very closely the male and female patterns found in donor control chicks of the same age.

The contour feather may be entirely donor colored or partly donor and partly host colored—i.e., mosaics of donor and host colors. Both types may appear on the same chick; in some, the mosaic type only occurs. There are several types of mosaic feathers. In the commonest of these the distal portion of the vane and shaft is the color of the donor, and the proximal portion is host colored (Figs. 21-23). The transition between these portions of the feather is quite sharp, forming a line roughly transverse to the shaft. The proportion of donor- and host-colored regions varies with the sequence in origin of the contour feathers. The amount of the donor-colored portion is greatest in the primary and secondary flight feathers which arise first and least in those arising later (Fig. 20). The amount may be still less in the coverts and breast feathers, which arise still later than the primaries and secondaries. The donor influence on color production which thus ceases before the emergence of the juvenile plumage is completed never reappears.

A second type of mosaic feather is found which differs from the one just described in that the transition between donor and host coloration is more gradual and somewhat irregular. A variable number of patterns is exhibited among the wing and breast feathers: (1) those with the shaft donor colored either throughout its length or distal portions of varying lengths; (2) feathers like the foregoing except for the bases of the barbs, which are donor colored also; (3) feathers with donor coloration extending in a proximal direction farther on one side of the vane than on the other (in these the transition zone is somewhat broad, donor and host coloration intermingling).

A third type of mosaic feather may be designated "alternating," since the donor- and host-colored areas in the vane tend to alternate. The donor color merely temporarily disappears and then reappears. The cessation in the formation of the donor color may take place once or several times during the outgrowth of the feather. A variety of patterns has formed among the flight feathers, wing coverts, and breast feathers of the juvenile plumage. The following few cases will illustrate the phenomenon. The first, a secondary flight feather (No. 1) has its distal one-fifth barred like the host (Plymouth Rock), while the remainder is white like the donor (Silkie Bantam); upon plucking, this feather was replaced by one entirely barred, like the host. A second example is that of an under wing covert. The distal one-fifth of this feather is donor (F_1 hybrid ♀); the

second fifth is host (White Leghorn); the third fifth, donor; the fourth fifth, host; and the proximal fifth, donor. A third case is that of a secondary flight (Nos. 1 or 10). In this the distal portion of the vane (half or fourth) is entirely donor (F_1 hybrid ♀); the middle portion, host colored (White Leghorn) except for the shaft and bases of the barbs, which are donor colored; the proximal portion, entirely donor-like.

A fourth type of mosaic feather found is unilateral with respect to color. The donor color is confined to one side, either on the inner or on the outer vane. In such a feather, shown in Figure 24, the entire inner vane and shaft may be mostly black, like the host (Black Minorca), and the outer vane entirely white, like the donor (Silkie Bantam); or the distal half of the inner vane (i.e., sixth primary) may be barred like the donor (Plymouth Rock) and the proximal half red like the host (New Hampshire Red), while the outer vane is entirely host colored. In some feathers (i.e., ninth secondary), the vane is entirely donor colored (White Silkie) except for the bases of the barbs of the inner vane, which are host colored (Black Minorca). In addition to these, other feathers are found in which the regions of donor color, although confined to one side, are smaller and of variable shape.

A microscopic examination of barbule cells from the downy region of the contour feathers shows, as did the down feathers, the presence or absence of pigment granules, depending upon whether the donor was from a pigmented or nonpigmented breed (see Figs. 26-37). When pigmented breeds, such as the Barred Plymouth Rock and the Black Minorca, are donors to White Leghorn hosts, the barbule cells contain black granules characteristic of these breeds. In Figure 33 White Leghorn host barbule cells containing pigment granules of the Black Minorca are seen. These granules are of the same form as those seen in the control Minorca cells. Similarly, when Barred Plymouth Rock or New Hampshire Reds are used, the granules occurring in the White Leghorn host are, respectively, of Barred Plymouth Rock and New Hampshire Red types (Figs. 27 and 30). Their distribution is the same in the host feathers as in the donor control feathers. When white breeds, such as Wyandotte, Silkies, and Plymouth Rocks, are donors, the barbs and barbules of the white host feathers, as in the donors, contain few or no pigment granules (Fig. 37). Likewise, White Leghorn implants in Black and Buff Minorca hosts may bring about the formation of a few white down feathers containing some pigment granules, more or less localized at their bases, as in control donors.

The various contour feathers developed in the "graft" area have the same type and arrangement as feathers in corresponding positions of host controls of the same age or on the unoperated wing (left) of the host (Fig. 4). Those on the wing are arranged in definite tracts containing the primaries on the hand; secondaries and coverts, etc., on the forearm. On the breast they have the distribution characteristic of the ventral tract. Their shape and size are always peculiar to the tract or a particular region of the tract in which they occur. In no case do the feathers of the donor-colored area resemble in shape and distribution the feathers of the head, limb, or trunk regions of the embryo from which the skin implant was taken. For example, head skin ectoderm, when implanted to the wing, does not replace host skin and produce feathers like those occurring on the head of control donor chicks—i.e., small feathers arranged in a number of small tracts. The feathers are entirely of the host wing type.

Likewise, the rate of growth of the donor-colored feather is that of the host. The daily rate of growth of certain primaries and secondary flight feathers has been measured and found to follow exactly that found for corresponding feathers on the unoperated left wing

(see Fig. 4) or on host controls of the same age. Figure 1 shows a graph in which the rate of growth in length of the fourth and fifth primaries, having the color pattern of the Barred Plymouth Rock donor, a slow-feathering breed, is found to be practically identical with that of the fourth and fifth primaries of the left wing of the White Leghorn host, a fast-feathering breed. The rate of growth in length of these feathers in a Barred Plymouth Rock control chick is distinctly slower than in the White Leghorn. Also, the third and fourth red primary feathers colored by a New Hampshire Red im-

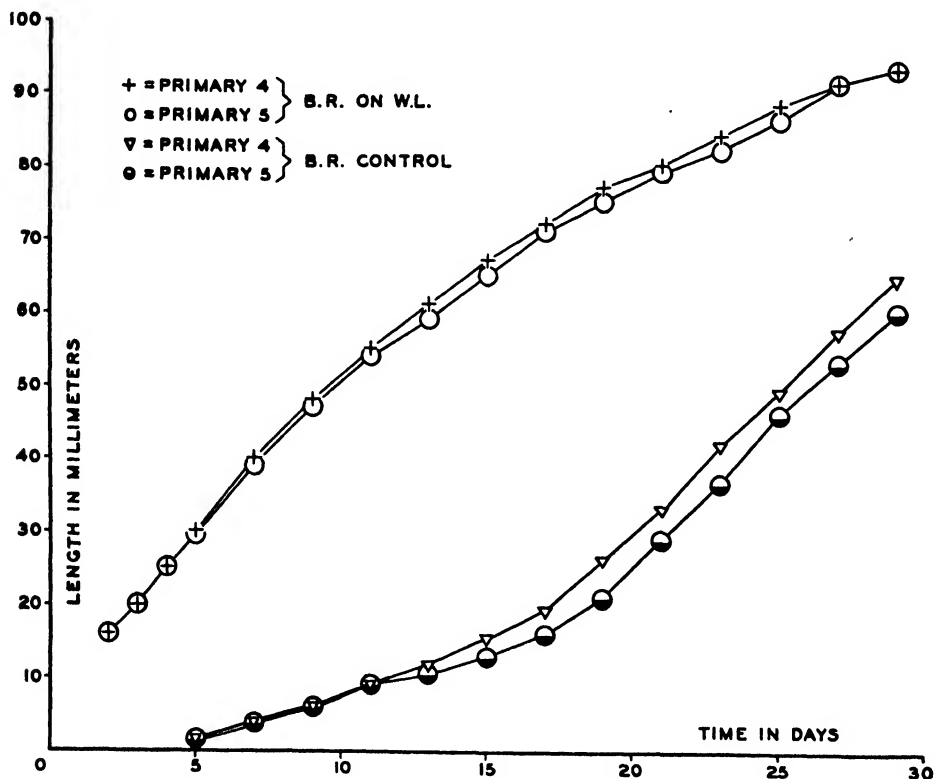


FIG. 1.—Comparison of the rate of growth in length of barred (donor pattern) primary flight feathers Nos. 4 and 5 of a White Leghorn host with that of the same feathers of a Barred Plymouth Rock control. The donor colored feather has the White Leghorn and not the Barred Plymouth Rock rate of growth.

plant on a Barred Plymouth Rock host develop at the Barred Plymouth Rock rate of feathering. In the control New Hampshire Red these feathers grow in length somewhat more rapidly than in the control Barred Plymouth Rock.

REPLACEMENT OF JUVENILE BY ADULT PLUMAGE

The donor-colored juvenile plumage of the "graft" area is gradually replaced with adult plumage as molting takes place. Usually with this molt the new feather which emerges is entirely host colored, the donor color having completely disappeared. The order in which this takes place in the flight feathers of donor color on White Leghorn

hosts is a very regular one, agreeing quite precisely with the order described by Warren and Gordon (1935) for the normal White Leghorn. The primaries are molted in regular sequence, beginning with No. 1, adjoining the axial feather, and progressing outward to No. 10. The secondaries are dropped in the reverse order, beginning with No. 2 and progressing inward. Secondary No. 1 and the axial are molted considerably later. The new adult flight feathers emerge in the same order in which the juvenile ones are dropped, but usually with the color of the host. The sequence in the shedding of the coverts and breast feathers was not followed.

In certain exceptional cases some of the primaries and secondaries are replaced by adult feathers which are mosaics of donor- and host-colored parts. In one feather (first primary) of the adult plumage of a White Leghorn chicken which received an implant of Barred Rock tissues, the outer vane has the Barred Rock pattern, whereas the distal half of the inner vane is entirely white (hostlike), and its proximal half is barred. The shaft of the feather is barred except the white half next to the white vane. Another feather (fifth secondary) is nearly identical with the one just described. Still another type of mosaic adult feather is shown in Figure 25, where the host is White Leghorn and the donor Black Minorca. Here the feather is entirely black, like the donor, except for two symmetrically arranged host-colored (white) areas in the inner and outer vanes at the tip (this is possibly an injury effect brought about by plucking; see Kuhn, 1932). A third type of mosaic adult feather has the donor color (black) largely confined to the shaft and the bases of the barbs of both outer and inner vanes. In some feathers the black pigment may extend the entire length of, or only part way down, the shaft. Toward the base of the feather the black pigment not only may be confined to the shaft but may occur on one side of it, the other side being white or host colored. Finally, it may be noted that, as far as our studies go, when such mosaic feathers are replaced the new (second adult) feathers are entirely host colored.

FATE OF THE IMPLANTED SKIN ECTODERM

Attempts have been made to follow the fate of the implanted head skin ectoderm and its adhering mesenchyme in the host embryo. At successive intervals after implantation, ranging from 5½ to 72 hours, the host embryo was prepared for histological study in the usual manner, and the right wing bud was carefully examined for the implanted tissue. Twenty-seven cases have been examined. In the majority of these, 21 in number, the host was White Leghorn and the implant Barred Plymouth Rock; in 4 cases the host was Barred Plymouth Rock and the donor White Leghorn; and in 2 instances Barred Plymouth Rock was host to New Hampshire Red tissue.

The results of this study show almost invariably at the implantation site the presence of a piece of the skin ectoderm of the implant adjacent to which is a mass of loose, light-staining mesenchyme. The piece lies deep in the limb mesoderm, especially during the early hours after implantation. It is usually continuous at the surface, with epidermis covering the original incision. How much of the surface epidermis is of host and how much of donor origin cannot be ascertained, owing to the absence of distinguishing features.³ The epidermis beneath the surface may be folded upon itself in the form of a

³ On the basis of the interpretation reached in this paper, a feather developing from any persisting epidermis would be structurally like the donor breed but colored according to whether the feather germ was invaded by donor or host melanophores. Of all the donor breeds tested, only the White Silkie has feathers distinguishable by structural features from the others. The Silkie type of feather has not been found in the "graft" area, indicating the absence of persisting donor epidermis.

vesicle, or its free end may terminate blindly in the wing mesoderm. The deeper portions of the skin ectoderm, particularly when entirely surrounded with mesoderm, become disorganized; its epithelial character is lost, and the cells intermingle with, and are indistinguishable from, the mesodermal cells of the wing bud. In a few instances the implant is situated near the surface of the wing and may protrude through the original incision and fuse with the amnion. In such cases it is inferred that the implant is in the process of being lifted out.

The mass of loose, light-staining mesenchyme of the "graft" region contrasts sharply with the dense, dark-staining limb mesoderm. The source of this mesenchyme is uncertain. It may arise from the mesenchyme introduced with the head skin or from both donor and host cells resembling a tissue akin to scar tissue in the adult. In no case can donor mesenchyme or other types of cells be identified.

It is apparent from this study that the implant remains localized at the base of the wing bud, although the limb has increased considerably in length. The growth of the wing bud is apparently entirely distal to the site of implantation. There is no evidence that the skin ectoderm of the implant spreads over the wing, replacing the host epidermis of the donor-colored feather.

Finally, it should be noted that there is no difference, so far as these studies go, in the behavior of White Leghorn implants on Barred Plymouth Rock from that found in reciprocal combinations of these breeds. The failure of the White Leghorn grafts to produce donor-colored feathers in F_1 -hybrid and New Hampshire Red hosts must therefore be explained on other grounds.

DISCUSSION

Manner of origin of the area of donor-colored feathers.—Both donor and host appear to play a role in feather characterization within the graft area. Structurally, the feathers are of host epidermal and not of graft origin. This is shown by several lines of evidence. First, the spread of the donor-colored effect over the entire wing and to adjacent feather tracts on the breast, back, and even to the thigh in some cases, is too extensive to regard the implant of skin ectoderm as the source of the epidermal cells of the feather germs. Second, a histological study of the fate of the skin ectoderm made at successive intervals after implantation reveals that it does not spread over the wing, replacing the host epidermis of the graft area, but remains localized at the base of the limb bud, the site of grafting. Apparently only a small surface portion heals in, becoming continuous with the surrounding host epidermis; the deeper portions of the implant become disorganized, the epithelial character of the ectoderm is lost, and the cells intermingle with, and become indistinguishable from, the mesodermal cells of the wing bud. The growth of the wing bud is apparently largely distal to the site of implantation.

In the third place, the donor-colored contour feathers of the juvenile plumage have the same shape, rate of growth, and distribution in tracts as those found in corresponding regions of host control chicks (see figs. 1 and 4). In these particulars they never resemble feathers of the head region, which might be expected, assuming that the donor epidermis retained its head qualities and produced them. Of particular importance in this connection are the results of implanting Silkie Bantam skin ectoderm into Black Minorca or into Barred Plymouth Rock hosts. In these cases, had the Silkie ectoderm formed the structural elements of the donor-colored feathers, such feathers would, barring the possibility of host effects, have possessed the defects characteristic of the donor breed, such as

missing barbicels in most feathers, frayed margins of the vane in the primary and secondary flight feathers, etc. Such was not the case. In every respect except color, the feathers which formed in the graft area were identical with host control feathers.

A fourth consideration is the fact that a donor-colored area of feathers produced by an implant of limb-bud mesoderm or somite mesoderm (including neural crest cells) (Watterson, 1938) has characteristics identical with one produced by an implant of skin ectoderm. In the former case the host epidermis undoubtedly forms the feather structure, since no donor ectoderm was transplanted.

Finally, conclusive evidence that the donor-colored feather arises structurally from host epidermis following skin-ectoderm implantation has been furnished by grafting Robin into White Leghorn, birds belonging to two widely separated orders (Rawles, 1938 and 1939). A microscopical examination of the proximal and distal barbules of the inner and outer vanes and of the downy barbules of Robin-colored contour feathers of the host shows that the feathers are definitely White Leghorn in structure. Only the color of the feather is Robin, owing to the presence of pigment granules of a specific Robin variety.

The color or color pattern of the feathers in the graft area, on the other hand, is produced under the control of the donor breed. In all the various combinations of breeds tested, the color or color pattern of the feather has been that of the donor breed. This is true irrespective of whether the donor belongs to a pigmented or unpigmented breed. The only exception to this rule occurs when White Leghorn is donor to F_1 -hybrid and New Hampshire Red hosts, in which cases white feathers fail to develop in the "graft area." For other peculiarities in the behavior of White Leghorn implants see page 181.

From these findings on the contribution made by donor and host, the interpretation is reached that the feather of the graft area is the product of the joint action of host feather germs and some influence arising from the implant of donor tissue. In other words, the structure of the feather is the product of the epidermal collar of a host feather germ, but its color or pattern is under the control of the implanted cells.

That the feather coloration is controlled in some way through the agency of melanophores derived from the donor was first suspected from the well-known fact that feather pigmentation in the fowl is associated commonly with the activity of melanophores in the epidermis of the feather germ. These, according to Strong (1902 and 1917), Greite (1934), and others, are branched cells which deposit or "feed" melanin pigment granules directly onto the surface (Greite) or into the interior (Strong) of the epidermal cells of the developing shaft, barbs, and barbules.

Several lines of evidence support the theory that the donor melanophores have actually migrated into the epidermis of the host feather germ, where they deposit pigment granules onto the barbs and barbules. In the first place, Watterson, working in this laboratory, has observed dermal melanophores entering the epidermis prior to the formation of feather germs in normal chick embryos of the Barred Plymouth Rock breed. Secondly, that melanophores do normally arise outside the epidermis and migrate into it is clearly shown by transplantation experiments. Our first clue as to their extrinsic origin came from experiments designed to test the capacity of skin ectoderm from various regions of the embryo to produce a donor-colored area in host embryos. It was noted for donors incubated less than 79 hours that skin ectoderm from the wing bud and from over the somites gave, respectively, negative and positive results. Later, after 79 hours, ectoderm from the wing bud, as well as from the somite region, gave positive

effects. Ectoderm from the leg bud did not give positive results until still later, i.e., after 100 hours. In a similar manner Watterson (1938) tested the pigment-producing potency of mesoderm cells from the limb bud and from somites. He found that the pigment-producing cells are present in the segmental plate and somites of 33–72-hour donors and appear in the wing bud sometime between 72 and 92 hours. He further showed that these pigment-producers appear still later in the leg bud—viz., in a donor of about 99 hours. From these two lines of results the conclusion is reached that (a) the source of the pigment cells is in the region adjacent to the neural tube, whence they migrate laterally into the limb buds and into overlying ectoderm, and (b) their outward spread is later in posterior than in anterior levels.

Eastlick (1939a and 1939b) reached a similar conclusion from a different type of analysis. He grafted a limb bud from 24–30 somite embryos of pigmented breeds (Brown Leghorn or Barred Plymouth Rock) to the coelom of White Leghorn or of pigmented host embryos of the same somite number and found that, if the bud was isolated so as to include all material (except entoderm) up to the neural tube, it developed pigmented feathers in the graft. If, however, the limb bud was isolated from the body at the level of the nephrotome, it produced down feathers without pigment. Thus, in the absence of pigment cells located in the region of the neural tube a feather germ of a pigmented breed can produce only white feathers.

Evidence of a similar nature is furnished by grafting wing buds of different ages to the coelom or to the chorio-allantois (Ris, 1940). A wing bud isolated from Barred Plymouth Rock embryos of 72-hours or earlier and grafted to these sites in either Barred Plymouth Rock (in case of coelomic grafts only if attached to mesentery) or White Leghorn hosts produces white down feathers only, no melanin pigment being formed. Similar transplantations of wing buds of older embryos (90 hours and over) give black down feathers in the graft. By means of a histological examination Ris showed further that melanophores are present in the epidermis of the feather germs of the latter but not in the former.

Lastly, crucial evidence that the donor melanophores have entered the epidermis of the host feather germ is furnished by the discovery of melanin granules of a shape, size, and color peculiar to the donor breed in the barb and barbule cells of the host feather. Thus, the conclusion is reached that the breed-specific melanophores have migrated away from the donor implant and have entered the feather germs of the host, where they deposit breed-specific granules.

The question next arises as to the source in the normal embryo of the melanophores which appear to migrate into the epidermis of the feather germs of limb buds and other body parts. The evidence discussed above on their extra-epidermal origin and lateral migration from the neural tube region, together with the work of Du Shane (1935, 1938) and others on the source of chromatophores in amphibians, leads to the hypothesis that the neural crest furnishes the melanophores in the chick. Reasoning from analogy, Dorris (1936, 1938, and 1939) was the first to suspect that the neural crest in the chick, as in the amphibian, embryo is the source of the melanophores. She consequently set about testing the capacity of the neural crest region in the chick to produce pigment cells. She first found that melanophores differentiate in explants of the neural crest region taken from embryos of several breeds of fowl. Later she showed that, when the neural crest is grafted to a host embryo, as in our experiments, it has the capacity to produce an area of donor-colored down feathers.

That the neural crest is the exclusive source of the epidermal melanophores has not been shown. The controls used by Dorris are inadequate for showing this, since little or no attempt was made to correlate pigment production in explants or in grafts with the order of appearance and development of the neural crest in the donor embryo. For example, ectoderm lateral to the somite or to the neural crest, if taken from one level in the anteroposterior axis, may give positive results, while from another level only negative results. Furthermore, the removal of the entire neural crest of an embryo is no assurance that pigment cannot form in isolates taken from levels of the axis where this structure has not yet formed. By transplanting parts from various levels of a Barred Plymouth Rock embryo into the coelom of a White Leghorn host embryo, Ris (1940) has shown that a definite correlation exists between the capacity of an isolate to produce pigment and the morphological development of the neural crest. Only those isolates known to contain a neural crest, the region from which it arises, or its migrating cells are able to produce melanophores.

Recently the pigment-producing capacity has been traced to presomite blastoderms, stages prior to the formation of the neural crest. Quite independently Eastlick (1939a) and Rawles and Willier (1939) tested pieces from various regions of blastoderms of the Barred Plymouth Rock breed for their capacity to produce donor-pigmented feathers in White Leghorn hosts when placed in the wing or into the coelom. It was found that the blastodermic area capable of producing donor-colored plumage in the host chick coincides with the area known to form nerve tissue (both central nerve tissue and ganglia) in chorio-allantoic grafts (see Rawles, 1936), being much more extensive than the prospective neural-crest region, as indicated by vital staining. For a more complete account see Rawles (1940). The wide distribution of the pigment-producing capacity characteristic of the presomite blastoderms becomes restricted to the neural-crest region in the somite stages. The time when this change takes place, as well as its manner, are problems of considerable significance.

Having shown that the implant is the source of the melanophores of the donor-colored area, the question now arises as to what factors control the degree, paths, and direction of their spread in the body of the host. In general, when placed at the base of the wing bud of a 70-75-hour host embryo, the path of their extension is toward the tip of the wing and ventral to, but usually not across, the mid-line of the breast; also, posteriorly onto the back and thigh and anteriorly to a much lesser extent. The spread, as a rule, does not extend dorsally across the mid-dorsal line. When placed into the tail bud, the melanophores become distributed in the tail and adjacent regions of the chick. It is evident, particularly for the wing level, that more or less definite paths are followed. That these are the same paths followed by melanophores in their migration outward from the neural crest in normal development seems probable. Although melanophores are migratory cells, some influence of an unknown nature appears to direct their movements along certain lines. Such a directive influence may be effective in causing them to leave the skin-ectoderm implant and enter the tissues of the host.

It is clear, from grafting experiments cited above, that the host melanophores do not enter the wing bud before approximately 79 hours. The donor melanophores, which are grafted prior to this time, consequently have an opportunity to migrate into the wing bud before those of the host come in. That they do migrate in and invade the developing skin and feather germs before the host melanophores do is shown by the result that feathers with the coloration of the donor are formed first. Apparently, at

first the donor melanophores occupy all the available places in the ectoderm of the skin and feather germs, thus excluding those of the host, which come in later. As the donor melanophores are used up during the process of pigmentation of the feather, they are replaced by those of the host.

The extent of the spread of these cells apparently depends roughly upon the quantity of melanophores introduced in the implant. In general, small implants, either of skin ectoderm or mesoderm, have a tendency to produce less spread than large ones, i.e., the spread produced by a small implant may extend onto the forearm or onto the breast tract, whereas that produced by a large implant may extend over the entire wing and to the mid-ventral line of the breast (see Figs. 5 and 6). Also, skin ectoderm from the region over a somite produces a more extensive spread than skin ectoderm from the head. Such a difference is expected on the basis of a time difference in the development of these two regions of the embryo. In the head, the older region, the neural crest, as such, has already disappeared; and the melanophores are somewhat widely distributed, being within most of the skin ectoderm and subjacent mesenchyme of the head (less mesenchyme adheres to the isolated skin ectoderm from the head; hence fewer melanophores may be carried over in the implant). In the somite region at the stages used, the neural crest cells lie immediately beneath the skin ectoderm. Here the potential melanophores are still concentrated.

The age of the host seems also to be a factor in controlling the degree of spreading. This is indicated by the results of a few preliminary experiments in which the host was from 1 to 2 days older than customarily used. Although implants of the usual size were used, the donor-colored area formed on the wing was generally smaller than in younger hosts. In some cases the donor pigment was in the skin but not in the feathers. From this result it is inferred that the conditions in the wing region of older hosts are less favorable for extensive migration and for migration along certain paths. At the time of implantation the melanophores of such hosts have, in all probability, migrated to some extent in the direction of their definitive positions in the wing bud. Also, it has been found that melanophores spread much less when implanted on the head than on the wing and leg buds of 72-hour host embryos (cf. Figs. 3 and 5). This is attributed to differences in developmental age, the head region having passed through the stage when conditions are most favorable for spreading.

Other conditions within the host, apart from age, affect the degree of migration of melanophores. The same kind of melanophore, implanted in nearly equal quantities to hosts of different breeds, generally shows less spread in Barred Plymouth Rock, White Wyandotte, and New Hampshire Red than in the White Leghorn or the Black Minorca hosts. In the latter two hosts the spread generally may be described as being very extensive. Furthermore, Barred Plymouth Rock melanophores in a White Leghorn host spread to the tip of the wing or onto the breast, whereas in normal control Barred Plymouth Rock embryos or chicks, these regions are covered with white down feathers, which later become pigmented and ultimately barred. The nature of these host differences which favor or restrict the extent of migration of melanophores is a problem for further investigation.

It is also possible that the different kinds of melanophores, owing to some peculiarity in their constitution, vary in their ability to migrate. This is indicated for the White Leghorn melanophore since, even though large numbers are implanted, the spread is

much less extensive, irrespective of the host used, than when other melanophores are introduced. For a further discussion of this matter see pages 193-97 ff.

Breed-specific control of color pattern in host feathers.—The color or color pattern of the feathers in the invaded area is specifically in accord with the genotypic composition of the donor breed. This is shown in various ways. (1) A Barred Plymouth Rock implant produces a barring pattern in host contour feathers of nonbarred breeds such as the New Hampshire Red, White Leghorn, and Black Minorca (see Figs. 12 and 13). Furthermore, two types of barring pattern occur, one being generally somewhat darker than the other. In the darker pattern the black bars are wider and darker than in the lighter one. These differences parallel closely sex-linked differences in plumage found in donor control chicks of the same age, where the feathers of the female are somewhat darker than those of the male. It becomes apparent, therefore, that a melanophore from a female donor which has only one gene for barring produces a darker-colored host feather than one from a male which has two genes. The sex of the host apparently has no effect on the result. A more complete analysis of this matter is proposed for a separate paper. (2) Implants from male and female F₁-hybrid embryos produce, respectively, barred and nonbarred (Fig. 20) contour feathers in a White Leghorn host, irrespective of its sex. (3) An implant of a New Hampshire Red brings about in White Leghorn or Barred Plymouth Rock hosts an irregular black-and-red pattern in the contour feathers such as occurs in donor control feathers (Fig. 14). (4) A Black or Buff Minorca implant produces in the contour feathers of White Leghorn or of Barred Plymouth Rock hosts a solid black or buff coloration (Figs. 15 and 16). (5) The white donor breeds, including the White Leghorn produce in certain cases white feathers in pigmented hosts (Figs. 11, 17, 18, and 19).

The color and color pattern appear to be directly controlled through the agency of melanophores. The melanophores from pigmented breeds deposit melanin granules peculiar to the donor into the epidermal cells of the shaft, barbs, and barbules of the host feather. These granules are of a specific shape, size, and color, being characteristic of those found in the donor control breeds (Pl. VII).

When Black Minorca and Barred Plymouth Rock furnish the melanophores, black rodlike granules, characteristic of these breeds, occur in the feather irrespective of the breed of host (Figs. 27, 28, 33, and 34). Buff Minorca melanophores deposit yellow-colored granules which are smaller and rounded in form. Grafts of New Hampshire Red give a result of special interest. The host feather has both red- and black-colored areas, as in the donor control chicks. In these areas are found, respectively, red (phaeomelanin) and black (melanin) granules, which differ strikingly in size and shape. The black ones are rodlike, resembling Barred Plymouth Rock granules, except for a slightly greater length. The red ones are small globular granules (Figs. 29-31). According to Ladebeck (1923), pigment granules identical with these are found in the red and black regions of feathers of the Rhode Island Red, a strain from which the New Hampshire Red was apparently derived. This observation suggests the presence of two specific types of melanophores in the New Hampshire Red.

If two kinds of melanophores have already differentiated prior to the formation of the feather germ, it will be necessary to assume the presence of some differential factors controlling their distribution to the red and black areas in the feather vane. It seems more probable that the melanoblasts, at the time of their invasion, are relatively un-

differentiated cells. They may have the dual capacity for differentiating into either red or black melanophores, the direction being determined by regional differences in physiological activity within the epidermal substratum of the feather germ. Whether such melanophores are stable and incapable of changing one into the other is an interesting question. Raper (1932) has shown that various intermediate substances are formed in the action of tyrosinase on tyrosine. The first visible product is a red substance, which in later stages becomes reddish brown and finally black melanin. Thus, a second hypothesis, less likely than the foregoing, is that the melanoblast differentiates into only one kind of melanophore, which is more or less labile and synthesizes either phaeomelanin or melanin granules, depending upon external conditions.

The evidence seems clear that all the white breeds used in this study possess melanophores which are of a type peculiar to the donor. An *in vitro* study of skin-ectoderm explants from embryos of these breeds shows, in every case, the presence of melanophores containing melanin granules (Hamilton, unpublished results; see Dorris, 1936 and 1938, for their occurrence in the White Leghorn). Also, we have observed them directly in isolated pieces of the skin and feather germs (chiefly at their bases) of embryos of these breeds at stages just before hatching (also see Ris, 1940). Reasoning from analogy with the results obtained with the pigmented donors, it would appear that melanophores from the white-breed donors do enter and occupy definite positions in the feather germs of the pigmented host. Owing, however, to some peculiarity in their genetic constitution, few or no melanin granules are deposited, with the result that the host feather is white (Fig. 37). An examination of the feathers of these breeds reveals usually a complete absence of pigment granules (see Figs. 35-37). The melanophores of the White Leghorn are shown to differ from those of White Wyandotte, White Plymouth Rock, and White Silkie in being much less capable of producing an area of white feathers in pigmented hosts (see pp. 193-97 ff.).

From the analysis made above, it is concluded that the melanophore is a breed-specific cell whose action in a foreign feather germ is in accord with its genotypic composition. Furthermore, its action in controlling feather color pattern is, to a considerable degree, independent of the foreign-host environment. Similarly, Twitty (1936) and Twitty and Bodenstein (1939) in the studies on *Triturus* have shown that the pattern of melanophore distribution is determined primarily by species differences in the pigment-forming cells themselves.

The extent to which the melanophore behaves as an independent system in the production of the color pattern in the host feather remains to be considered. What factors peculiar to the host substratum, i.e., the epidermal collar of the feather germ, or humoral agents affect or modify the behavior of the melanophore? Important evidence on this question has come from a preliminary study of the patterns produced by melanophores derived from Barred Plymouth Rock, F_1 -hybrid, and Robin donors. The pigment pattern formed as a result of barred implants exhibits considerable variation in width of the black and the white bars. In general, irrespective of the sex of the donor contributing the melanophores, these bars are wider in rapidly growing feathers, such as the wing primaries, and narrower in slower-growing feathers, such as the upper wing coverts and breast feathers. This is particularly convincing in those cases where variations in the width of the pattern occur on the same host, even though the melanophores all came from the same donor and the same region of its body. Also, in certain cases, i.e., Barred Plymouth Rock on New Hampshire Red host, or male F_1 -hybrid on White

Leghorn host, some of the primary flight feathers show little or no white barring (trace on shaft only in some), whereas the late-emerging secondaries and upper wing coverts have a distinct barred pattern. It is thus apparent that rate of growth of the host feather germ is a factor in affecting barring pattern (cf. Montalenti, 1934). The production of a Robin-colored pattern in feathers of a White Leghorn host illustrates how the action of the Robin melanophore may be altered by the epidermal substratum in the feather germ of the host. In such feathers the apical end of the vane is reddish, and the remaining portion gray in color. For reasons similar to those given above in discussing the production of red-black color pattern in the New Hampshire Red, it is probable that these apicobasal differences in Robin coloration are under the control of regional differences in physiological activity within the host feather germ. The Robin melanoblast, under conditions peculiar to the apical portion of the feather germ, becomes a melanophore capable of producing red-pigment granules; whereas, in more proximal portions, it becomes a melanophore capable of forming black-pigment granules.

The distribution of the Robin-pigment granules in the down barbule cells of the host appears also to be under the control of the host epidermis. They are distributed more or less uniformly on the surface of the barbule cell, as is characteristic of fowls in general, and are not grouped at the nodes of the barbule, as occurs in the normal Robin (see Rawles, 1939, Figs. 9-13). A study of the feather germ for the origin of the structural differences found in the feather parts of the Robin and fowl may shed some light on how the distribution is effected. Differences in structural pattern within the feather germ may be of such a nature as to bring about a spacing or orientation of the melanophores and their processes peculiar to these two orders of birds, with the result that differences in pigment deposition and distribution to the epidermal cells occur.

It is well known from the work of Danforth (1933*a*), Lillie and Juhn (1932), and others, that humoral agents, such as thyroxine and sex hormones, may play a role in determining the kind of pigment pattern formed in the feather of adult plumage. The extent to which these agents modify or supplement the action of the melanophore in pattern production should be analyzed in the light of these experiments and conclusions.

Peculiar nature and behavior of the White Leghorn melanophore.—This investigation brings out clearly that the melanophores of the White Leghorn have a unique behavior in grafts. Implants containing White Leghorn melanophores produce an area of donor-colored feathers much less frequently and more limited in extent than do melanophores from any of the other white or any of the pigmented breeds tested. Implants to Black and Buff Minorca hosts give an area of white feathers with the highest frequency, which, however, is limited to the middle and distal portions of the wing. At first, implants of White Leghorn melanophores into Barred Plymouth Rock hosts yielded only negative results, but recently a few cases have been obtained in which a small area of white feathers did develop. So far, no positive results have been obtained on hosts of either New Hampshire Red or F₁-hybrid breeds. A similar peculiarity in the behavior of the White Leghorn melanophore in pigmented hosts has been noted by Dorris (1939), who found that they bring about the formation of white feathers on Australorp but fail to do so on Rhode Island Red hosts.

The question naturally arises as to the significance of this unique behavior of the White Leghorn melanophore when introduced into foreign hosts. Can it be correlated in any way with the peculiar genetic constitution of the White Leghorn race of fowls? This breed, according to the cross-breeding experiments of Hadley (1913, 1914, and

1915) and others, possesses (1) a white plumage character, which is dominant over dark color (for incomplete dominance over red and yellow see Danforth, 1933*b*) and (2) usually genic factors for black pigmentation and for barring. To explain the appearance of white in a bird with such genetic makeup, it has been generally assumed by poultry geneticists, following the suggestion of Hadley (1913), that an "inhibiting factor" is present which suppresses the formation of black pigment, not only in the feathers of the White Leghorn, which would be otherwise black, but also in the feathers, except for an occasional blackening, of crosses with black breeds. Beyond the conclusion that inherent black coloration is not expressed, no definite information is available concerning the nature of the mechanisms involved in this apparent inhibition.

Can this failure to produce black feathers be attributed to certain peculiar genetically controlled potentialities of the melanophores, or of the epidermal substratum, or to the combined action of these two components of a feather germ? The suggestion has been made that the epidermis contains a factor, chemical in nature, which prevents the melanophore from depositing pigment granules. The ability of the melanophore to produce pigment *in vitro* and *in vivo*, but inability to deposit pigment in the feather germ, appears to support this view. It has been observed that, when transplants are made to Leghorn hosts of older stages (i.e., at 5 days), the donor-colored area is generally smaller than in younger hosts. Does this indicate that the inhibitor factor is making or has made its appearance in the epidermis, as Dorris (1939) has suggested? The fact that an area of reduced dimensions is likewise produced, as a rule, when barred melanophores are grafted to New Hampshire Red hosts older than those usually employed (the number of negative cases also increases) makes this somewhat improbable. In all probability the formation of a smaller area in older hosts means that the conditions, whether mechanical or otherwise, suitable for the migration of the donor melanoblasts at the earlier stages are no longer favorable. Furthermore, the host melanoblasts have already migrated into the skin ectoderm before the donor ones are introduced; consequently, the latter have few or no available positions. Under such conditions the donor melanoblasts would be less effective in producing donor-colored feathers.

The strongest evidence against the view that the epidermis of the White Leghorn has a means for preventing pigment deposition is furnished by the general results of grafting. If an inhibitor factor were present in the epidermis, it would be expected that melanophores from pigmented donors would not be able either to produce or to deposit pigment granules in the White Leghorn feathers. Such is not the case, since all pigmented donors tested, very readily produced an area of pigmented feathers in the White Leghorn host. In no single case is the action of a melanophore from any pigmented breed of fowl, or even from a Robin, inhibited. It produces a color hue or a color pattern just as faithfully as it would in corresponding feather germs of donor control chicks.

Sooner or later, however, the donor pigment cells, irrespective of their source, apparently become exhausted, whereupon the feather becomes white or host colored. This usually takes place some time during the emergence of the juvenile plumage (see Fig. 20), but occasionally not until the adult plumage appears. Apparently, this does not indicate the onset of any new physiological conditions within the feather germs of the White Leghorn peculiar to it, since donor pigmentation becomes exhausted in an identical manner in hosts of all other breeds tested. In all probability, its disappearance is correlated with the quantity of melanophores originally introduced in the graft. If

implants containing a part of the neural crest where the potential melanophores are apparently concentrated are introduced, the effect may persist even into the adult plumage—many months after the operation (as long as 18 months in one case). With implants containing less melanophores, i.e., head skin ectoderm (see p. 182), the effect may persist only during the formation of the apical ends of the juvenile contour feathers.

In this connection it is important to inquire why, in general, the donor melanophores fail to persist throughout the life of the host, especially since there is no apparent incompatibility between host and donor melanophores. Obviously, the melanophores are being used up continuously in the growing feather of the host, and new ones must be forthcoming from some source as the new feather germ organizes, following each molt. A superfluous supply should be available, since no host melanophores were removed at the time of grafting. It is probable that a special zone of the follicular epithelium, described by Lillie and Juhn (1938) as "regenerating cells," is the source of the reserve melanophores. A special study of this zone in a regenerating feather may shed some light not only on their source but also on why the melanophores of the donor are sooner or later replaced by those of the host. It is, of course, conceivable that long-continued residence in a foreign host may alter the melanophores physiologically, so that ultimately they are more easily supplanted by those of host origin, or that an immunity reaction is built up against them.

This replacement of the donor with host melanophores in these experiments is in marked contrast to results obtained by Danforth (1937 and 1939) in grafts of skin made after hatching. He noted only a slight tendency for the melanophores of the grafted skin to be replaced by those of the host, even after several natural and induced molts. Conditions fundamentally different in the two methods of grafting may account for this. When the transplantation is made early in embryonic life, the only foreign element in the host skin is the donor melanophore; whereas, after hatching, the donor furnishes the dermis and the epidermis, as well as the melanophores. When this difference is considered, the results are much less surprising.

Having argued, from the available evidence, that the epidermis of the White Leghorn has no properties essentially different from those of other breeds which could explain the lack of pigmentation, we shall now turn to the melanophores themselves for a possible clue. It was first thought that their ability to produce white feathers in Buff and Black Minorca hosts could be attributed to some constitutional properties possessed by these hosts and not by any of the others tested. This seemed particularly convincing when it was noted that those hosts giving positive effects are rapid feathering, while those giving negative results are slower-feathering races. Since, however, the white-feathered area was smaller in the Minorca hosts, much in contrast to the large areas produced by White Silkie implants in hosts of the same breed, it next occurred to us that these results might be explained, at least in part, on the hypothesis that the melanophores of the White Leghorn (*a*) are quantitatively fewer or (*b*) have less ability to migrate than in other breeds. In a preliminary study of this matter it has been found that pieces of skin ectoderm, including subjacent cells of the neural crest from the White Leghorn, will occasionally produce a definite area of white feathers on a Barred Plymouth Rock chick, a host heretofore always negative (see Fig. 11). The white area is of limited dimensions, however.

Apparently only when large numbers of potential melanophores are introduced can the donor effect be produced in the Barred Plymouth Rock host, and even then it is limited

in its extent. It cannot be decided from this result alone whether the donor melanophores are merely few in number or somehow limited in their powers to migrate, or whether both conditions are concerned. Observations on living feather germs of the White Leghorn indicate that the number of melanophores containing black pigment may be relatively fewer than in other breeds (Ris and Hamilton). Also, their number apparently varies greatly from individual to individual. On the basis of a limited migratory power, especially of a retarded rate of spread, the host melanophores would be expected to reach the host epidermis before the donor ones do, with the result that no donor effect is produced. This could explain the absence of the donor effect commonly found. A still more puzzling problem is why they apparently reach their positions in the epidermis more readily in a fast-feathering, than in a slow-feathering, host. This matter of the rate and extent of spreading of the melanophores of the White Leghorn breed—and, indeed, of all breeds—furnishes a problem for further investigation.

The restricted power of the White Leghorn melanophores to produce an area of white feathers in pigmented hosts, whether due to limited numbers or to some weakness in migration tendency, points to the interpretation that the melanophores themselves are responsible for the peculiarity in behavior noted. This viewpoint is strongly supported by the demonstration that the Leghorn feather germ exhibits no tendency whatsoever to prevent melanophores of colored breeds from forming and depositing pigment in it. Moreover, from an analogy with the action of melanophores of other breeds, where each acts primarily as an independent system in producing color or color pattern, it is reasonable to conclude that this peculiarity must be attributed largely to the action of the White Leghorn melanophore itself. Like all the other melanophores examined, its genetic constitution apparently fixes its ability to act and react in the foreign host environment.

Another striking characteristic of the White Leghorn melanophore is its limited capacity to deposit pigment. An examination of a White Leghorn feather germ shows the presence in the epidermal collar of normally branched melanophores containing black-pigment granules. As the parts of the feather arise from the collar, the melanophores, as a rule, withdraw their processes, round up, and die. The result is that few or no pigment granules are deposited in the epidermal cells of the feather parts. Apparently the melanophores of this breed usually have, at first, the capacity to synthesize black melanin pigment; but, as they grow older, some change of a lethal nature takes place which prevents them from forming additional pigment or from depositing that already formed.

This peculiar behavior is apparently an intrinsic property of the melanophore. This is brought out very nicely in a study of its action in the Minorca and Barred Plymouth Rock hosts. In the feather germs of these hosts, where normally pigment granules are readily deposited, it can produce white feathers. Even under conditions favorable for pigment synthesis and deposition, it still lacks the ability to deposit pigment. Apparently, these pigmented hosts do not supply to the White Leghorn melanophore the factor necessary for pigment formation and deposition.

In the White Leghorn itself, the ability to deposit pigment in the feathers is apparently not controlled by the epidermal substratum, since, as noted above, the latter does not hinder the synthesis and deposition of pigment granules on the feathers by melanophores of any breed thus far grafted. The melanophore thus appears to be quite

independent of the epidermal substratum. This furnishes additional support for the view that the limitation of melanin deposition is primarily under endogenous control.

The extent to which the melanophore of this breed is independent of exogenous conditions is a question of some significance. Such external factors as enzymes, humoral agents, and the like must be recognized as playing a possible role in modifying the expression of some of the genetic potentialities of these cells.

SUMMARY

1. This report deals with the control of feather color pattern by grafting a small piece of skin ectoderm or pure mesoderm from one embryo to another of the same age (67-108 hours' incubation) but of a genetically different breed. The graft site was usually the base of the right wing bud; but other regions, such as the leg and tail buds and the head, were sometimes used.

2. Isolates from the head or trunk regions, at all ages used, usually produce an area of donor-colored down feathers on the host at and about the site of grafting, often extending to adjacent regions. Skin ectoderm or mesoderm from the wing bud of embryos younger than 80 hours or from the leg bud earlier than 96 hours fails to produce such an area.

3. An area of donor-colored feathers has been obtained in all host-graft combinations tested between two pigmented and between pigmented and unpigmented breeds. The White Leghorn, in contrast to all other donor breeds, produces a very restricted area of donor-colored feathers and with a lower frequency.

4. The donor-colored down feathers are gradually and normally replaced by contour feathers of the juvenile plumage having the color or color pattern of the donor breed; but as regards shape, size, and distribution in tracts, they are identical with corresponding host control feathers.

5. The donor-colored plumage is ultimately replaced by host-colored feathers. If this occurs during the emergence of the juvenile or adult feather, it becomes a mosaic of donor and host coloration. Usually the donor effect disappears with the molting of the juvenile plumage, but occasionally it persists in some of the feathers of the first set of adult plumage. The time of disappearance of the donor coloration seems to be roughly proportional to the quantity of melanophores brought in by the implant.

6. The feathers of the donor-colored area are structurally of host epidermal origin. This is shown by (a) the failure of the skin-ectoderm implant to replace the host epidermis, (b) the production of donor-colored feathers with grafts of pure mesoderm, (c) the development of structurally normal (non-Silkie) contour feathers in Black Minorca or Barred Plymouth Rock hosts following the implantation of Silkie skin ectoderm, and (d) the formation of Robin-colored contour feathers of typical fowl structure by grafting Robin-skin ectoderm to the White Leghorn.

7. The feather coloration is controlled through the agency of melanophores, which migrate from the implant into the host feather germ, there depositing specific melanin granules of the donor type in the epidermal cells of the developing feather.

8. Evidence is presented to show that melanophores migrate shortly after 80 hours into the wing, and after 96 hours into the leg, from an outside source.

9. The neural crest as the exclusive source of melanophores is discussed. In presomite blastoderms the capacity to form melanophores coincides with the neural-forming area.

10. The action of the melanophore in controlling color or color pattern in the host feather is in accord with its genotypic constitution, i.e., faithful production of donor color pattern and deposition of donor-specific melanin granules in the host feather and is highly independent of the foreign host environment.

11. Melanophores from the white breeds apparently enter and occupy definite positions in the feather germs of colored host breeds. Because of some peculiarity in genetic constitution, they deposit few or no melanin granules, with the result that the host feather is white.

12. The peculiar behavior of the White Leghorn melanophores in grafts is an intrinsic property since (1) the epidermis of a pigmented breed does not change their limited power to synthesize and deposit melanin and (2) the epidermis of the White Leghorn never inhibits melanin synthesis and deposition by melanophores from pigmented breeds.

13. Finally, the extent to which the melanophore behaves as an independent system in the production of color pattern in the host is considered. Extrinsic factors (e.g., growth-rate of feather germ, humoral agents, etc.) are shown to play a role.

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PLATE I

FIG. 2.—Photograph of a living 72-hour host chick embryo showing a piece of stained skin ectoderm grafted to the base of the right wing bud. \times ca. 5.

FIG. 3.—A 5-day-old White Leghorn chick exhibiting an area of black down on top of the head. Produced by grafting to the hindbrain region of the host embryo at 79 hours a piece of skin ectoderm from the base of a wing bud of a Barred Plymouth Rock donor embryo of the same age.

FIG. 4.—A 22-day-old New Hampshire Red chick showing barred plumage on the right wing. Produced by grafting to the wing-bud base of the host embryo at 82 hours a piece of head skin ectoderm from a Barred Plymouth Rock embryo of the same age.

PLATE II

FIG. 5.—A 1-day-old ♂ White Leghorn chick showing area of black down feathers (first few primary flight feathers partly emerged) covering entire wing and part of breast. Produced by grafting to wing-bud base of the host embryo at 78 hours a piece of head skin ectoderm from a Barred Plymouth Rock embryo of the same age.

FIG. 6.—Same chick as the one shown in Fig. 5, 18 days after hatching.

FIG. 7.—Same chick as those shown in Figs. 5 and 6, 31 days after hatching. Note barred pattern in contour feathers of wing and breast.

PLATE III

FIG. 8.—A 4-day-old Black Minorca chick showing an area of white plumage covering the entire wing and right side of breast. Produced by implanting into the base of the wing bud of the host embryo at 74 hours a piece of head skin ectoderm from a White Wyandotte embryo of the same age.

FIG. 9.—A 23-day-old Barred Plymouth Rock chick showing white plumage on the right wing. Produced by grafting to the wing-bud base of the host embryo at 70 hours a piece of head skin ectoderm obtained from a White Silkie bantam embryo of 71 hours' incubation.

PLATE IV

FIG. 10.—A 34-day-old ♀ White Leghorn chick showing solid black contour feathers on the right wing. Produced by grafting to the wing-bud base of the host embryo at 73 hours a piece of head skin ectoderm from a Black Minorca embryo of the same age.

FIG. 11.—Tip of the eighth primary and six upper coverts of a 1-month-old Barred Plymouth Rock chick showing solid white coloration. Produced by transplanting to the wing-bud base of the host embryo at 78 hours the neural crest region (including upper part of neural tube and somite and overlying skin ectoderm from posterior level of body) of a White Leghorn embryo of the same age.

PLATE V

All feathers shown are remiges of the juvenile plumage of the host chick.

FIG. 12.—Tenth secondary of a White Leghorn host showing a barred pattern produced by melanophores from a Barred Plymouth Rock implant (from same chick shown in Fig. 7).

FIG. 13.—Seventh primary of a New Hampshire Red host showing a barred pattern produced by melanophores from a Barred Plymouth Rock donor.

FIG. 14.—Seventh primary of a White Leghorn host showing a red-and-black (to left of shaft) pattern produced by melanophores derived from a New Hampshire Red implant.

FIG. 15.—Eleventh secondary of a White Leghorn host showing solid red coloration produced by melanophores from a Buff Minorca implant.

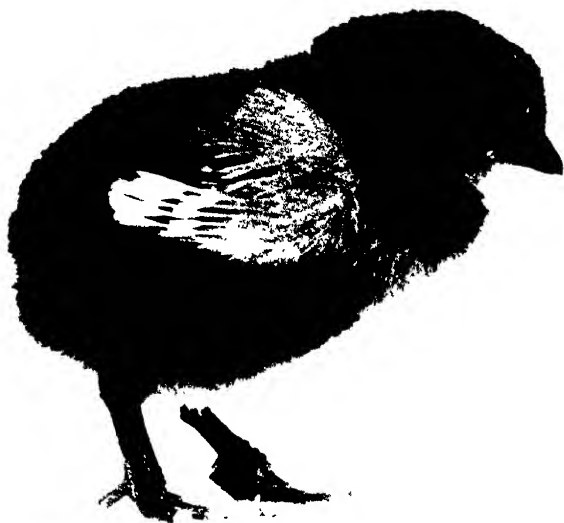
PLATE I



PLATE II



PLATE III



8



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PLATE IV

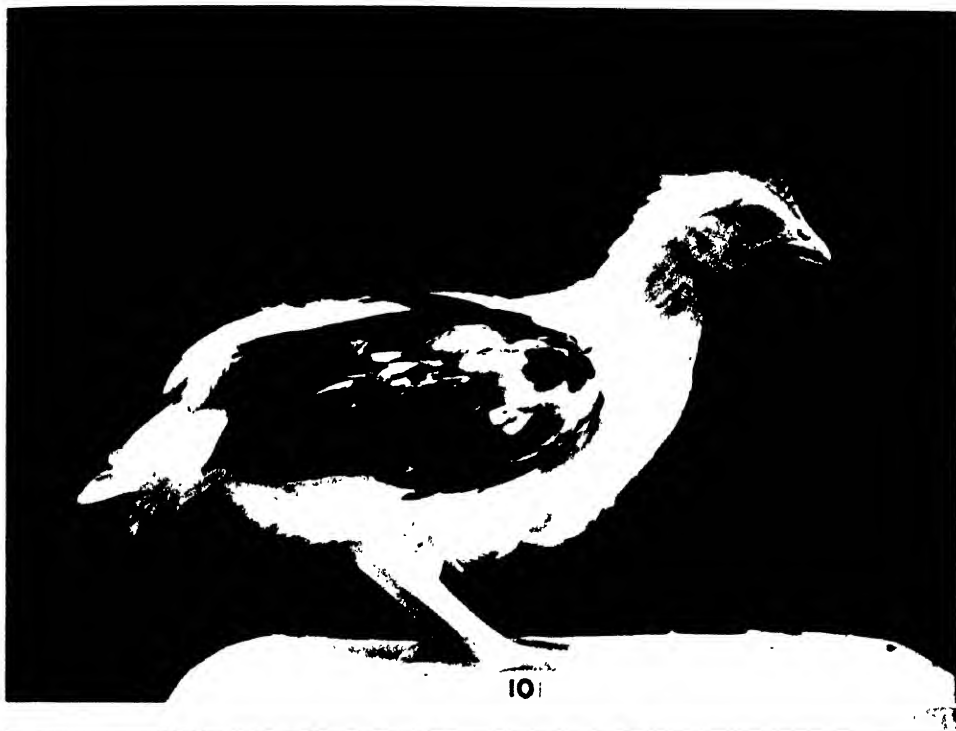


PLATE V

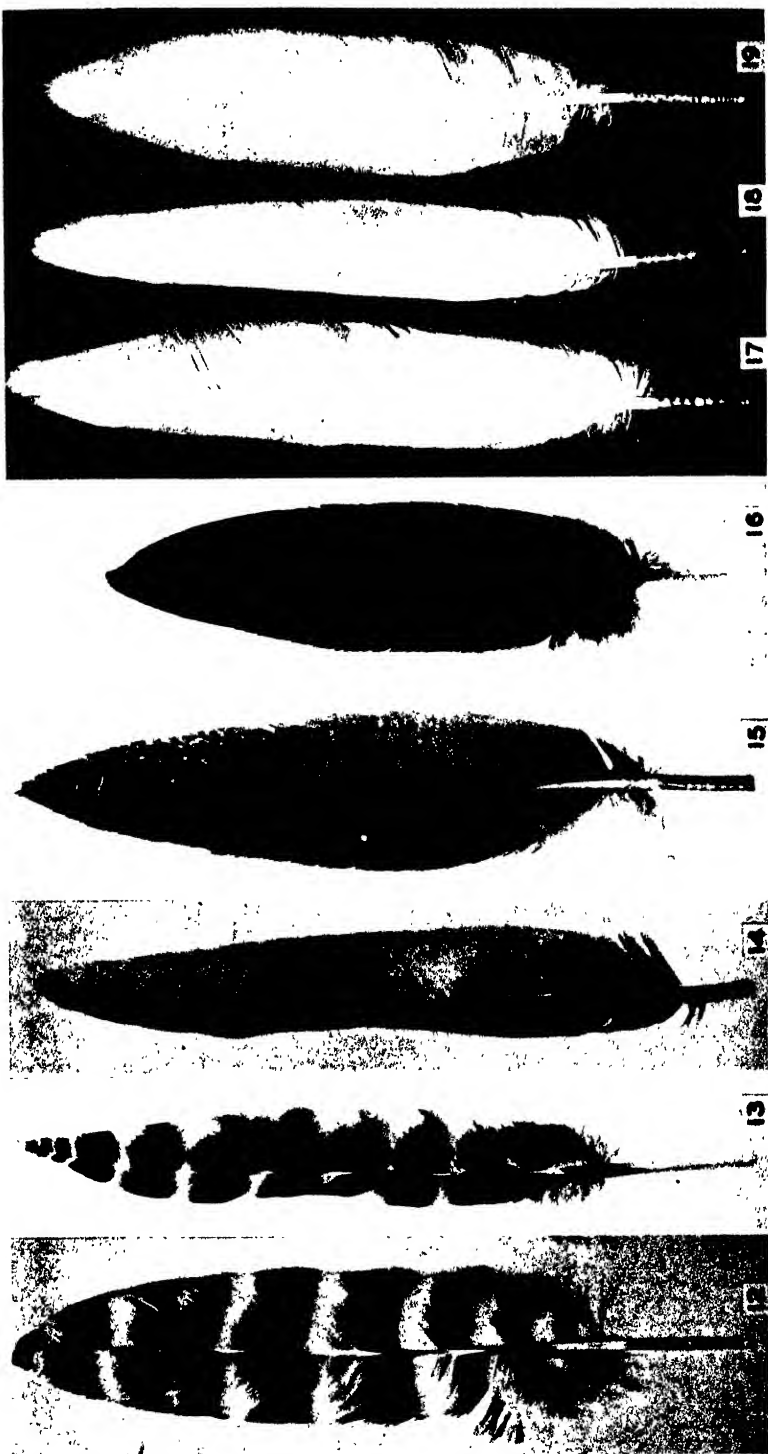


PLATE VI

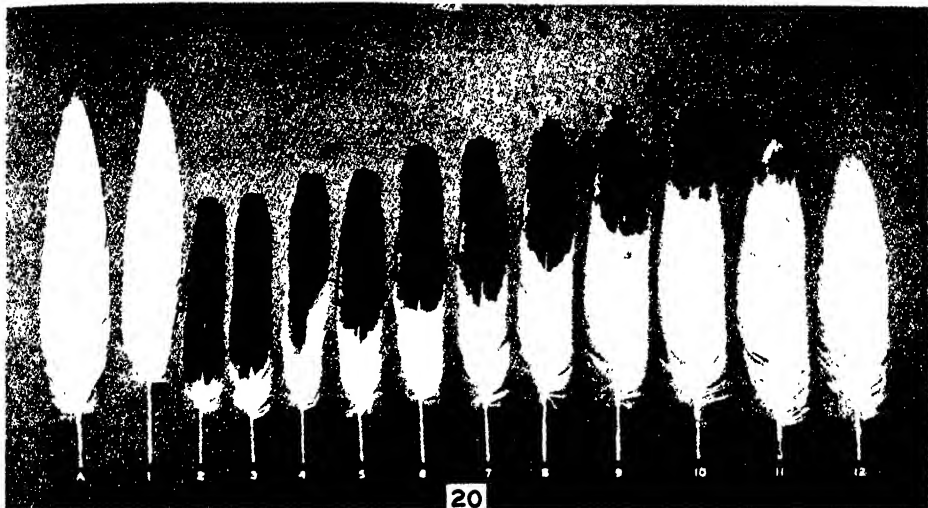
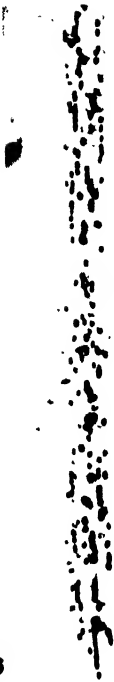


PLATE VII



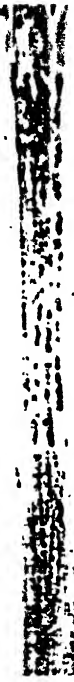
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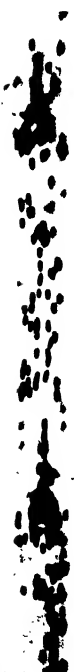
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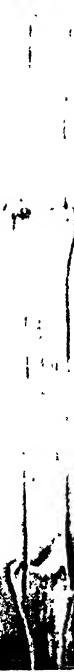
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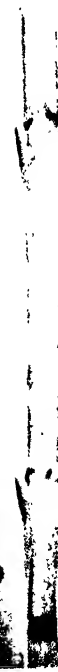
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FIG. 16.—Axial feather of a Barred Plymouth Rock host showing solid black coloration produced by melanophores from a Black Minorca implant.

FIG. 17.—Second primary of a Barred Plymouth Rock host showing solid white coloration produced by melanophores from a White Wyandotte implant.

FIG. 18.—Fifth primary of a Black Minorca host showing solid white coloration produced by melanophores from a White Silkie implant.

FIG. 19.—Third secondary of a Barred Plymouth Rock host showing solid white coloration produced by melanophores from a White Silkie implant.

PLATE VI

All feathers, except as noted, are remiges from the juvenile plumage of the host chick.

FIG. 20.—Axial (A) and secondary (1-12) feathers of a White Leghorn chick which received at 74 hours' incubation an implant of head skin ectoderm from an F₁ hybrid ♀ donor. The extent of hybrid pigmentation produced in a feather varies directly with the time of its emergence, being greatest in the first feather (No. 2) and least in the last ones to emerge. The time of disappearance of pigmentation in the various feathers is approximately simultaneous.

FIG. 21.—Seventh secondary from a New Hampshire Red host. The apical two-thirds has the barred pattern of the Barred Plymouth Rock donor; and the proximal third, the black-and-red pattern of the host.

FIG. 22.—Seventh primary from a Barred Plymouth Rock host. The apical one-fourth has the black-and-red pattern of the New Hampshire Red donor; and the proximal three-fourths, the barred pattern of the host.

FIG. 23.—Third primary from a Barred Plymouth Rock host. The apical half is white, like the White Wyandotte donor; and the proximal half has the barred pattern of the host.

FIG. 24.—Third secondary of a Black Minorca host. The left half of the vane is white, like the White Silkie donor; and the right half is mostly black, like the host.

FIG. 25.—Tenth secondary of the first adult plumage of a White Leghorn host. Outer portions of the vane at the tip are white, like the host; the remainder, solid black, like the Black Minorca donor.

PLATE VII

Photomicrographs of unstained whole mounts of barbules from the downy region of covert and flight contour feathers, with the exception of Fig. 31, which comes from the down plumage. \times ca. 810.

FIG. 26.—Melanin granules of Barred Plymouth Rock control.

FIG. 27.—Melanin granules of Barred Plymouth Rock deposited in White Leghorn.

FIG. 28.—Melanin granules of Barred Plymouth Rock deposited in New Hampshire Red.

FIG. 29.—Melanin granules of New Hampshire Red control.

FIG. 30.—Melanin granules of a New Hampshire Red deposited in White Leghorn.

FIG. 31.—Melanin granules of a New Hampshire Red deposited in Barred Plymouth Rock.

FIG. 32.—Melanin granules of Black Minorca control.

FIG. 33.—Melanin granules of Black Minorca in White Leghorn.

FIG. 34.—Melanin granules of Black Minorca in Barred Plymouth Rock.

FIG. 35.—White Leghorn control showing absence of pigment granules.

FIG. 36.—White Silkie Bantam control showing absence of pigment granules.

FIG. 37.—Black Minorca barbule showing absence of pigment granules when White Silkie is the donor.

THE LOCALIZATION OF PIGMENT-FORMING AREAS IN THE CHICK BLASTODERM AT THE PRIMITIVE- STREAK STAGE¹

(Three text figures and one plate)

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RECENTLY a number of investigators have attacked problems relating to the production of pigment by embryonic chicks. In 1937 Willier, Rawles, and Hadorn made skin grafts between different breeds of chick embryos and found that a piece of ectoderm from the head of a potentially colored embryo had the capacity to produce a pigmented area when grafted to the wing bud of a noncolored host. In a later contribution Willier and Rawles (1938b) reported that a piece of limb-bud mesoderm from a colored donor would also produce the same result.

Dorris (1936, 1938a, 1938b, 1939) was able to show that neural crest cells from a colored embryo produced melanin not only when transplanted to the limb bud of a colorless host but also when cultivated *in vitro*.

Eastlick (1938) corroborated Dorris' conclusion that pigment cells are derived from the neural crest by making reciprocal limb-bud transplants between embryos of pigmented and nonpigmented breeds. He found that the pigment-forming cells of 56-72-hour Brown Leghorn or Barred Plymouth Rock embryos were concentrated along the neural tube. By varying the position of the cut made in removing the limb-bud primordium of a potentially colored embryo, it was possible to secure a nonpigmented, a partly pigmented, or a fully pigmented graft when the bud was transplanted to a White Leghorn host. These results indicated that the pigment-producing cells migrate peripherally from the neural tube and later occur in the ectoderm and mesoderm.

The present study was made in an attempt to gain information concerning the localization of pigment-forming areas in pre-somite chick embryos. Accurately measured portions of blastoderms of Barred Plymouth Rock embryos in definitive primitive streak stages (13-20 hours of incubation) were transplanted to White Leghorn hosts which possessed between twenty-six and thirty-three somites. The presence or absence of pigmented feathers on the host was a criterion of whether the grafted area included cells which were able to differentiate into melanophores.

A demonstration of this work was given at the spring meeting of the American Association of Anatomists in Boston (Eastlick, 1939a). At that time Rawles and Willier (1939) reported a paper on a similar subject. While the blastoderms were sectioned at different levels in the two laboratories, and slightly different procedures were followed in grafting the pieces to the hosts, the results seem to supplement each other and appear to warrant the same general conclusions.

EXPERIMENTAL PROCEDURES

The following procedure was used in making the operations. The egg which contained the donor embryo was opened after sterilizing the shell with alcohol, and the

¹ This investigation was aided by a grant from the Research Council of the University of Missouri.

region of the primitive streak was stained by means of an agar block which contained neutral red. The blastoderm was then severed from the yolk, removed to warm, sterile salt solution, and washed free of adhering yolk granules. The blastoderm was flattened by pipetting away most of the fluid and then examined under a dissecting microscope.

TABLE 1

SUMMARY OF RESULTS OBTAINED WHEN VARIOUS PIECES OF PRIMITIVE-STREAK BLASTODERMS OF BARRED PLYMOUTH ROCK EMBRYOS WERE GRAFTED TO THE LATERAL BODY WALL OF WHITE LEGHORN EMBRYOS

SERIES A

	PIECE GRAFTED (SEE FIG. 1)			
	1	2	3	4
Pigment produced	3	18	0	0
No pigment produced	20	5	19	13
Died before ninth day	9	12	12	10
Total	32	35	31	23

SERIES B

	PIECE GRAFTED (SEE FIG. 2)				
	A	B	C	D	E
Pigment produced	2	12	13	0	0
No pigment produced	15	5	5	17	14
Died before ninth day	8	10	5	7	9
Total	25	27	23	24	23

SERIES C

	PIECE GRAFTED (SEE FIG. 3)										
	1	2	3	4	5	6	7	8	9	10	11
Pigment produced	1	2	0	3	8	1	2	9	1	0	0
No pigment produced	11	10	9	10	1	10	6	2	13	10	7
Died before ninth day	5	6	7	5	7	7	9	7	5	8	5
Total	17	18	16	18	16	18	17	18	19	18	12

Measurements of the length of the streak, the distance from the primitive pit to the anterior edge of the area pellucida, and the width of the area pellucida at the pit level were made by means of an ocular micrometer. An outline of pieces of the desired size was made by means of a glass needle, according to the method described by Rawles (1936).

In preparing the host, an opening 5-7 mm. square was made in the shell directly over the embryo. The shell membranes were removed in the region of the opening, and the body wall of the embryo was stained with an agar block saturated with neutral red. The vitelline membrane was slit, and the amnion torn open whenever necessary. A small incision was made in the body wall just posterior to the wing bud and slightly lateral to the posterior cardinal vein. A full description of this procedure has been given by Hamburger (1933, 1938).

The position of the cuts that were made in separating the blastoderm into pieces may be ascertained by referring to Figures 1-3. In all cases an attempt was made to remove the mesentoderm from the mesectoderm. The removal was made quite easily in the pieces anterior to the node; but in the region of the streak, the close adherence of the layers usually prevented their complete separation.

The desired piece of blastoderm was drawn up with a Spemann pipette and transferred to the lateral body wall of the host. It was oriented within the incision by means of a glass needle. As the wound healed, the piece of blastoderm became incorporated in the body wall of the host. In some cases the transplanted portions were pushed through the incision into the intra-embryonic coelom and oriented over the vitelline artery rather than being left within the slit in the body wall.

All hosts were allowed to develop 15 or more days. The majority were sacrificed between 17 and 19 days, while a few were allowed to hatch.

OBSERVATIONS

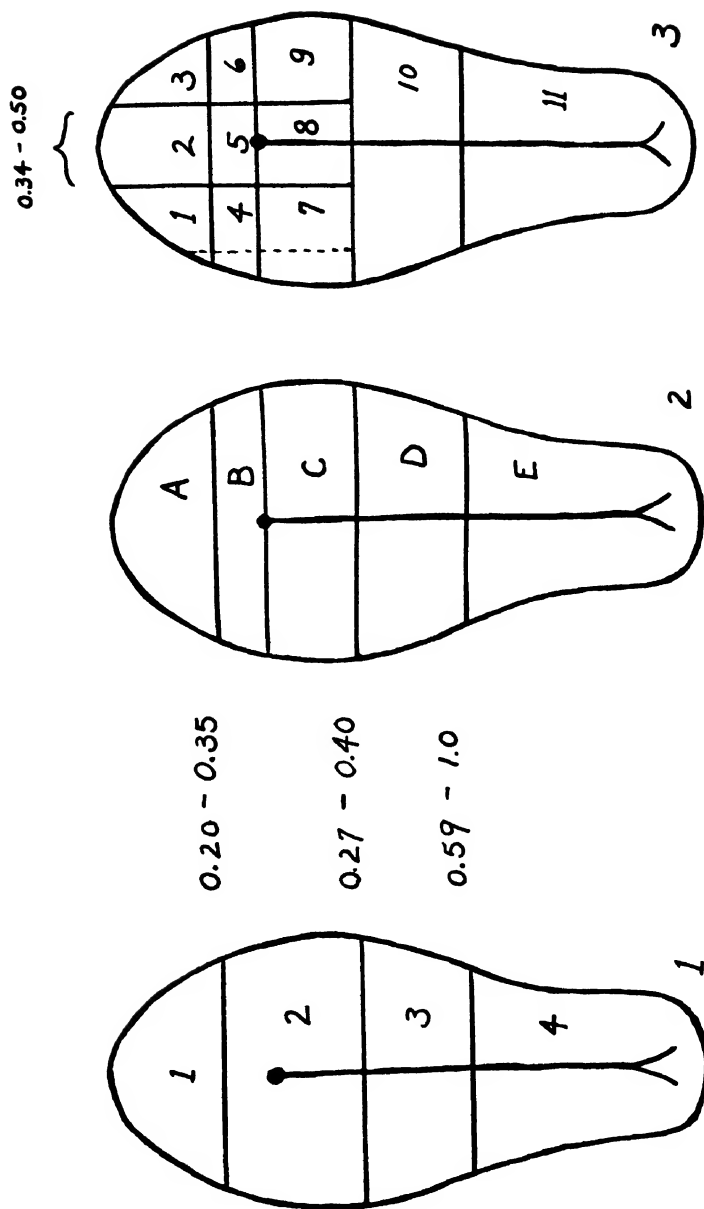
Series A.—Table 1 presents the summary of the results which have been obtained in the three series of experiments. In the table the grafted pieces are designated in the same manner as in Figures 1-3. The measured limits of the grafted pieces may be noted by referring to the diagrams.

The most anterior piece in Series A developed pigment in approximately 13 per cent of the cases (see Table 1, Ser. A). Even in the positive cases the pigmented areas were small and possessed feathers which were only partially pigmented. In some of these mosaics a portion of the feather was colored, and the rest of it was nonpigmented; while in others the pigment was laid down irregularly throughout the whole feather, so that a streaked appearance resulted.

Piece 2 in Series A developed pigment in 18 out of 23 surviving cases, or in approximately 78 per cent of the grafts. A large area of pigmented feathers usually occurred in these hosts. In one case the entire lateral body wall, the wing, and the breast of the host became intensely pigmented. The melanophores migrated from the point of the incision and invaded a large surrounding area. Moreover, a few of the transplants which were made into the intra-embryonic coelom developed into good-sized masses which bore colored plumage (Fig. 4).

An examination of the positive cases reveals that variations occur in the size of the colored patches and in the degree of coloration of the feathers. This may be associated with the degree of incorporation of the graft, its viability, etc. It is significant, however, that the portion of the blastoderm which contained the node invariably produced a higher percentage of positive cases than other regions.

Series B.—The second set of experiments was designed to test whether a transverse strip of blastoderm containing the anterior half of the node has greater capacity to produce melanophores than a piece containing the posterior half of the node. The re-



FIGS. 1-3.—Diagrams which illustrate the location of cuts made in the blastoderm. The measurements (in millimeters, represent the distance from the primitive pit. Blastoderms sectioned as in Fig. 1 constitute Series A; as in Fig. 2, Series B; etc

sults which have been obtained are inconclusive, since the number of successful cases is approximately equal in both cases (see Table 1, Ser. B). Both anterior and posterior pieces produced pigment in approximately 70 per cent of the hosts which lived long enough to allow one to make certain of the presence of colored feathers. An examination of the hosts has failed to reveal any significant differences either in the intensity of the pigmentation or in the extent of the area which is colored (Figs. 5 and 6).

The results which have been obtained by grafting the anterior piece *A* and the posterior pieces *D* and *E* are strictly comparable to those obtained in Series A. Two positive cases only were secured from grafts of piece *A*. In each of these a small area was pigmented, and many of the feathers were mosaics. The posterior pieces gave negative results in all cases.

Series C.—This series was made in an endeavor to locate within rather close limits the regions of the blastoderm which differentiate pigment cells. A summary of the results obtained in these experiments is shown in Table 1, Series C. Reference to the table reveals that the pieces which are cut 0.2–0.35 mm. anterior to the node have a restricted pigment-forming capacity. While the number of experiments is not large, the results suggest that the left anterior piece 1 has a higher potency than the right anterior piece 3 and that the middle section has the greatest potency of all. Even in the positive cases the pigmented area is small (Fig. 7), and the feathers are partly colored.

Pieces 4, 5, and 6 are strips, 0.2–0.35 mm. in width, which extend anteriorly from the mid-portion of the node. Each of these pieces has the capacity to differentiate pigment cells, but the number produced by the three pieces varies greatly (see Table 1). The left lateral piece 4 produced colored patches of feathers in 23 per cent of the cases. In two of the three positive cases a small area of colored down was produced; in the other, a fairly extensive pigmented area occurred. The center piece 5 which included the anterior half of the node, produced pigment in approximately 90 per cent of the cases. Not only was a higher percentage of positive cases secured from this piece than from any other portion of the blastoderm, but a larger pigmented area usually resulted. Moreover, most of the feathers are intensely pigmented. Only a single positive case has been secured from the right lateral piece 6. A small number of pigmented feathers occurred on this host (Fig. 8).

These results suggest that the left lateral pieces have a greater potency than the right lateral portions of the blastoderm. This same result was procured in pieces from the next level, namely, pieces 7, 8, and 9. The lateral pieces 7 and 9 produced approximately the same percentage of positive cases as pieces 4 and 6. Moreover, the extent of the pigmented areas is approximately the same in these pieces. Piece 8, which contained the posterior portion of the node, was able to develop pigment in approximately 80 per cent of the cases. No significant differences were noted in the intensity of the coloration or in the size of the pigmented areas produced by the anterior and posterior portions of the node. While the former produced a slightly higher percentage of positive cases, the difference does not seem to be statistically significant. The results show, beyond doubt, that the node produced more melanophores than other regions of the blastoderm.

As in Series A and B, the two posterior pieces failed to produce pigmented feathers. In one case a nodule which possessed noncolored feathers was formed on the lateral body wall at the point of the incision.

Forty-one operations were made in which pieces of blastoderm, sectioned as in Figure 3, were placed into the intra-embryonic coelom of the hosts. Of the four positive cases secured, three were transplants of piece 5, the other of piece 8. The blastoderm is very fragile, and apparently the majority of pieces disintegrate before receiving an adequate blood supply. Since such a small number of positive cases was secured, this method was discontinued.

So far, the earliest stages found to produce pigment were transplants of the middle third of 8-hour blastoderms. Two cases out of 12 were positive. A small patch of pigmented feathers resulted in each case. These results, while preliminary in nature, suggest that the blastoderm earlier than the definitive stage has relatively restricted pigment-forming capacity. It seems probable, however, that positive cases can be secured from all stages, including the unincubated blastoderm, since Butler (1935) has obtained nervous tissue in pieces of unincubated blastoderm transplanted to the chorioallantoic membrane, and Dalton (1935) has found medullary tissue in transplants of pieces of 8-hour blastoderms. A much larger number of experiments, however, will have to be made before any general conclusions can be drawn concerning the capacity of these early stages to develop pigment.

DISCUSSION

The foregoing experiments demonstrate that various regions of the blastoderm of Barred Plymouth Rock embryos at definitive primitive streak stages possess striking differences in their capacity to produce melanophores. Hensen's node seems to have the greatest ability to produce pigment. This is shown by the fact that pieces of blastoderm containing the node produce more positive cases than fragments from other regions. Moreover, an extensive heavily pigmented area usually results from such grafts. The pigment cells spread from the point of the incision and feed melanin into the developing follicle cells of the host.

When the node was bisected transversely, no significant differences were found in the pigment-forming capacity of the two halves (Figs. 5 and 6). The results are not strictly comparable, since the anterior was smaller than the posterior piece. Probably the position assumed by the blastoderm within the incision is of greater significance than the size of the grafted fragment. For example, the smaller piece may be placed in the slit in such a way that a greater percentage of its cells becomes incorporated in host tissue than may be the case with the larger strip. In the latter instance a considerable portion of the transplant may disintegrate, and only those cells develop which heal into the wound. Some of the most pronounced cases of pigmentation were the result of the transplantation of the smallest piece (5 in Ser. C).

The lateral pieces of the blastoderm 4 and 6, 7 and 9, have the capacity to produce melanophores, but the potency is much more restricted than in the region of the node. Probably the majority of melanophores arising from these pieces were developed in those portions which lie nearest the node. Twelve experiments have been made in which pieces of the blastoderm, cut 0.5-0.6 mm. to the left of the pit (regions lateral to the broken line in Fig. 3), were transplanted to the body wall of embryonic hosts. Negative results were secured in all cases.

Rawles (1936), in a study of the potencies of pieces of blastoderm as revealed by transplantation to the chorioallantoic membrane, found that the greatest number of positive cases were secured in fragments which included the node or were directly ad-

jacent to it, and that the percentage of "takes" diminished with increasing distance from this point. It is possible, therefore, that the ability of the different pieces of blastoderm to produce pigment may be associated with their capacity to become incorporated and to survive and grow. For example, the small number of pigmented cases secured after transplantation of the most anterior or lateral pieces may be due to the poor survival of these fragments rather than to their inherent inability to produce pigment. While this possibility cannot be excluded, the fact that some of these fragments gave rise to nodules which bore noncolored plumage is opposed to such an interpretation.

The localization of the pigment-forming areas noted in this study is essentially the same as that shown by the researches of Rawles and Willier (1939). They sectioned the blastoderm at levels slightly different from those used in this study; but, in general, very close agreement seems to have been reached concerning the localization of the melanophore-forming areas at the primitive-streak stages.

Investigations of the organization of the primitive-streak blastoderm of chick embryos have been made by injuring or vital-staining localized areas, by explanting pieces to the chorioallantoic membrane, or by culturing them *in vitro*. The results secured by these various methods have indicated that Hensen's node and the regions immediately surrounding it have the greatest capacity to produce medullary tissue. The various workers, however, are not in entire agreement regarding the exact limits of the area concerned.

Hunt (1932) reported that pieces of blastoderm cut more than 0.3 mm. anterior to the node possessed little capacity to develop neural tissue when transplanted to the chorioallantoic membrane. Rudnick (1938a), using tissue-culture methods, has substantiated these results. Apparently, the lateral limits of the area which produces the medullary plate have not been investigated extensively. Hunt (1932) reported that medullary tissue developed in very few transplants of pieces of blastoderm cut more than 0.3 mm. lateral to the pit. The studies of Wetzel (1929) and of Pasteels (1936) seem to substantiate this conclusion. A number of reports have appeared concerning just how far posterior to the pit neural tissue may be differentiated. Hunt (1931, 1932), using the chorioallantoic technique, reported that he was unable to secure central nervous tissue from pieces of the blastoderm cut more than 0.21 mm. posterior to the pit. Wetzel (1929) and Pasteels (1936) found that the ectoderm as far back as one-half the distance from the pit to the posterior end of the streak contributed to the formation of neural tissue.

Dalton (1935), by the use of chorioallantoic grafts, and Waddington (1935), by *in vitro* methods, showed that pieces of blastoderm at least 0.6 mm. posterior to the pit formed medullary plate. Rudnick (1938a, 1938b) cultured carefully measured pieces of the blastoderm and found that pieces more than 0.4 mm. posterior to the pit failed to develop central nervous tissue.

The foregoing data seem to indicate that an area extending 0.3 mm. anterior to the pit, 0.3 mm. or more lateral, and at least 0.4 mm. posterior to it possesses the maximum medullary-producing capacity at the definitive primitive-streak stage. The results obtained in the present study suggest that this same area is concerned with the production of pigment.

DuShane (1934, 1935) has shown that the neural crest produces melanophores in *Amblystoma*, and Dorris (1936, 1938a, 1938b, 1939) demonstrated that neural-crest

cells of young chick embryos produce pigment when cultured *in vitro* and when transplanted to 3-day chick embryos. Eastlick (1939b) has corroborated this latter observation and also has demonstrated that limb buds of 60-70-hour Barred Plymouth Rock embryos develop noncolored plumage when cut free at the level of the posterior cardinal vein and grafted into the coelom of host embryos. Limb buds of $3\frac{1}{2}$ -4-day embryos, similarly removed, invariably develop pigmented down. Apparently this is due to the migration of the neural-crest cells into the limb bud at the latter stages.

It is apparent that the pigment-forming area of the blastoderm, before the development of the neural crest, exceeds its prospective value. However, with the appearance of the crest, pigment formation seems to be taken over entirely by this derivative of the nervous system.

SUMMARY AND CONCLUSIONS

1. This study has revealed that various pieces of the blastoderm of Barred Plymouth Rock embryos in the definitive primitive-streak stage differ greatly in their capacity to produce melanophores when transplanted to the lateral body wall or into the intra-embryonic coelom of White Leghorn embryos.

2. Each transplant was found to possess a specific developmental potency which manifests itself in (a) the frequency of pigment production, (b) the extent of the pigmented patch, and (c) the intensity of pigmentation of the colored area.

3. It has been demonstrated that the region which contains the node possesses the maximum ability to produce pigment. Portions of the blastoderm more than 0.35 mm. anterior, or 0.25 mm. lateral, to the pit possess restricted potency, as shown by the fact that few positive cases were secured, and the pigmented areas were small. All pieces of the blastoderm 0.4 mm. or more posterior to the node give negative results.

4. The results suggest that the pigment-forming area coincides with the regions which have been shown by Hunt, Rawles, Rudnick, and others to differentiate medullary tissue in chorioallantoic grafts or in explants.

5. Few successful grafts were secured when pieces of the blastoderm, large or small, were placed into the intra-embryonic coelom.

6. In two cases the middle third of 8-hour blastoderms has been found to produce small pigmented areas.

While this paper was in press, a report entitled "The pigment-forming potency of early chick blastoderms" by M. E. Rawles appeared in the *Proceedings of the National Academy of Sciences*, 26:86-94, 1940. The author studied the pigment-producing capacity of unincubated blastoderms and stages through eight somites. The results which she obtained with fragments of primitive streak stages seem to be similar to those found in the foregoing report. Her experiments show conclusively that the pigment-producing areas of early chick blastoderms coincide with the areas which develop nervous tissue in chorioallantoic grafts.

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PLATE I



PLATE I

FIG. 4.—Piece 2 in Series A transplanted into intra-embryonic coelom. Note large mass loosely attached to mesenteries bearing colored plumage. Transplants of same region grafted to lateral body wall usually produced large heavily pigmented areas.

FIG. 5.—Piece B in Series B transplanted to body wall. Large pigmented area resulted. Node region consistently produced extensive heavily pigmented areas.

FIG. 6.—Piece C in Series B transplanted to body wall. Posterior half of node consistently pigmented many feathers of host.

FIG. 7.—Piece 2 in Series C transplanted to lateral body wall. Middle pieces of blastoderm consistently produced higher percentage of positive cases and more extensive colored areas than lateral pieces of the same level.

FIG. 8.—Piece 6 of Series C transplanted to body wall. A few pigmented feathers resulted. Right lateral pieces seemed to have lower potency than corresponding left portions.

FIG. 9.—Piece 7 of Series C transplanted to body wall. Rather large pigmented area shown. The other positive case possessed a smaller colored area.

THE EFFECT OF EXCESS POTASSIUM ON THE CORTEX OF THE FERTILIZED EGG OF *ARBACIA PUNCTULATA*

(Two plates)

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IT seldom becomes possible to study a single reaction system of protoplasm inside the living cell. However, one of us (Moser, 1939*a* and 1939*b*) has recently succeeded in doing this for the cortical-layer granules of the unfertilized *Arbacia* egg. He was able to show that all sorts of stimulating agents (sperm, mechanical, radiational, electrical, and chemical) cause these surface granules to break down in a characteristic response leading to elevation of the fertilization membrane. Here, then, one possesses an extremely reactive protoplasmic system whose biochemical and physiological properties can to some extent be studied.

It is the purpose of this paper, the first of a series, to report upon another protoplasmic system available for experimental attack. This consists of the red-pigment granules of the living, fertilized *Arbacia* egg. The function of these granules is not known. One very interesting feature of their behavior has been remarked upon by several observers. This is the fact that the chromatophores, which lie freely distributed in the interior of the unfertilized egg, become localized in a differentiated cortex after having migrated peripherally to this region within 10 minutes (monaster stage) after fertilization (Harvey, 1910). This peculiar cytological behavior of the chromatophores is paralleled by their susceptibility to certain chemicals. It will be shown that certain reagents cause these granules to "explode" and to release the pigment from the living, intact egg. However, these reagents are not effective on the red granules in the unfertilized egg or, indeed, on those of the fertilized egg until about the time of the formation of the monaster.

It is therefore obvious that, even as the reaction of the surface granules reflects the properties of the cortex of the unfertilized egg, so the reaction of the red-pigment granules may be used to interpret the biochemical properties of the fertilized egg cortex. Fortunately, in spite of our ignorance of the physiology of the chromatophores, we do possess some information concerning the chemical conditions which cause these granules to become unstable and to disintegrate. Heilbrunn (1934) has shown that they release their pigment in the presence of free calcium or magnesium ions (surface precipitation reaction), calcium being perhaps one hundred times as effective as magnesium. When eggs are crushed in isotonic solutions of sodium, potassium, or calcium—binding agents, like citrate or oxalate—the red chromatophores remain intact. In other words, the breakdown of the chromatophores may be used as a criterion of the presence of free calcium or magnesium ions. We assume, therefore, that when a reagent like potassium (which, as noted above, has no effect on the isolated pigment granules) causes the chromatophores to break down in the living egg, it does so in an indirect manner, namely, by releasing calcium or magnesium from bound combinations. As previously

noted, the breakdown of the chromatophores takes place only after they have become definitely localized in the outer stiff cortex of the cell. This fact is additional evidence that it is the cortex which contains the masked calcium compound.

METHODS

Two methods have been employed to demonstrate the effects to be described. One is macroscopical; the other, microscopical. In general, the microscopical method, being much more difficult, was used but seldom. It serves as a check on the observations made by the macroscopical technique. In any case, certain precautions must be observed. Thus, it is necessary to avoid contamination of the egg suspension by the yellow perivisceral fluid. For this reason eggs should not be procured by permitting the urchins to shed, but the ovaries should be removed from the test. The ovarian tissue must be discarded immediately after the eggs have been collected. The egg suspension is then filtered through a double thickness of fine cheesecloth.

The experimental reagents consisted of three isotonic potassium salts. These were 0.53 M potassium chloride, 0.35 M potassium citrate, and 0.25 M and 0.30 M potassium oxalate. The isotonicity was tested by immersing the eggs in these solutions and observing that neither shrinking nor swelling occurred.

In both the macroscopical and the microscopical methods the proportion of reagent to egg suspension was alike, namely, 1:1. The macroscopical method was carried out as follows. Into the first of a dozen small test tubes, set in a rack, was placed a pipette—full of a moderately thick suspension of unfertilized eggs. To this was added an equal volume of one of the experimental solutions listed above, and the tube was inverted once to insure mixing. The color of the supernatant fluid could be determined almost immediately. The preparation of the second tube was carried out in the same way, except that the eggs had been fertilized. Such a tube could be prepared 1 or 2 minutes after fertilization. Five minutes later (i.e., about 7 minutes after insemination) a third tube was set up; then a fourth (12 minutes after fertilization); and so on. The last two or three tubes were prepared with eggs in the two-, three-, or many-celled stage, or even blastulae. The entire series was then photographed in color with the help of Mr. E. P. Little.

The microscopical ("drop") method consists in first placing a very small drop of egg suspension on a slide without adding a cover slip. A single egg was quickly selected for study with the high dry objective. To the edge of this drop was added an equal volume of the experimental solution. This diffused rapidly throughout the system, as was evident from the diffusion and convection currents set up around the egg that was being observed. The resultant changes of the red-pigment granules within the cell were recorded by photomicrographs and by a motion-picture apparatus kindly placed at our disposal by Dr. D. L. Hopkins, of Duke University, to whom we are greatly indebted for this service. Observations were made under light- and dark-field illumination.

OBSERVATIONS

If one carries out the macroscopical procedure outlined above, using 1 part of isotonic potassium salt to 1 part of egg suspension, one gets a series of colorless and colored supernatant fluids, as illustrated in Plate I. At the bottom of each test tube may be seen the layer formed by the eggs that have settled. The first tube (starting on the left)

contains unfertilized eggs, and the supernatant fluid is colorless. The next three test tubes also show colorless supernatant fluids. These contain fertilized eggs: the first prepared 2 minutes; the second, 5 minutes; and the third, 8 minutes, after fertilization. All the other tubes (the fifth set up 11 minutes after fertilization) show "orange" supernatant fluids. It is important to note that the color appears *instantaneously*; it is apparent even as the eggs are settling after inverting the mixture.

If, now, one examines the colored supernatant fluid under a microscope, it is found to consist of a clear transparent solution *which never contains any cytolized eggs*. In other words, the color is due to the release of the echinochrome pigment from the red granules of the living egg. Examination of the fertilized eggs in the KCl-sea-water mixture within an hour or two reveals (at the height of the season) 95-100 per cent first and second cleavages. Citrate and oxalate usually inhibit the cleavage of eggs left for so long a time in the tubes, although there can be no doubt that there is no toxic effect within the first 15-30 minutes. This must be so, since all the eggs reach the diaster stage. As a matter of fact, this toxic effect is no doubt in some measure due to CO₂ accumulation, because eggs immersed in less dense suspensions show a much higher percentage of successful cleavages.

The foregoing effects are substantiated by the "drop" method. When a drop of 0.53 M KCl is added to a drop of unfertilized eggs under the microscope, nothing happens. Nor is there a reaction if the KCl solution is added to eggs 5-8 minutes after being fertilized. But, if a drop of the KCl solution is added to eggs that are in the monaster stage (about 12 minutes after fertilization), a striking phenomenon occurs. At this time the red-pigment granules have reached the cortex (Harvey, 1910; Brown, 1934). As one focuses on this cortical layer while the KCl solution is diffusing to the egg, a sudden "explosion" of these granules occurs, and they disappear from view. This is shown in Plate II, which represents a series of motion-picture frames of the exploding red-pigment granules of a single, centrifuged, fertilized egg. (The disappearance of individual red granules can easily be made out by concentrating one's attention on the groupings in the hyaline area. Even a casual glance at the hyaline areas of photomicrographs 1 and 33 shows the sequence of disappearances.) It is important to note that the pigment always escapes to the exterior from the living egg and is never trapped within it, as under conditions of so-called "pale" cytolysis. An important feature of this reaction is the fact that only a few chromatophores break down when the eggs are first immersed in the experimental solution. As a matter of fact, examination of eggs in a KCl-sea-water mixture at the fourth or fifth cleavage reveals the fact that there are a few red granules still present in the blastomeres. They seem to be especially stable when imbedded in the cleavage furrow region.

It may be noted that none of the salts is effective on the chromatophores of the unfertilized egg. This is true even if the cells are permitted to remain an hour or more in the experimental solutions. On the other hand, the ovarian tissue itself may give a slightly colored supernatant fluid. For this reason contamination must be assiduously avoided.

Furthermore, it appears that up to about 10 minutes after fertilization pigment is not released by these reagents. This phase of nonsusceptibility, however, is not repeated during the first 10 minutes of the second, third, or successive cleavages. Once the new fertilized egg cortex is established, the pigment can be released by these agents

at any time during early development. In oxalate the color can be recognized even through the great crop of white CaC_2O_4 crystals that forms as soon as the potassium oxalate is added.

DISCUSSION

As stated in the introduction, it is the purpose of this paper to describe a reaction which is capable of revealing the presence of free calcium ions in the cortex of the living, fertilized *Arbacia* egg. That this reaction is restricted to the cortical region of the cell is based upon the following evidences. Isotonic KCl has absolutely no effect upon the pigment granules of the unfertilized egg. Furthermore, this solution has no effect upon the pigment granules of fertilized eggs before the formation of the monaster (about 10 minutes after fertilization). In both these cases the pigment granules are distributed uniformly throughout the cell interior. However, once the fertilized egg has reached the monaster stage, isotonic KCl in sea water becomes effective in breaking down the chromatophores. The important thing to note here is the fact that at this time the pigment granules are no longer freely dispersed in the interior of the cell but have now become almost entirely localized in a new cortex. Bearing in mind the fact that potassium has no effect on the isolated pigment granules, we are led to the conclusion that the KCl solution reacts with the "ground substance" in which the pigment granules are imbedded, rather than directly on the pigment granules themselves.

Concerning the evidence for calcium release by the potassium ion, we have already presented the qualitative aspects as ascertained from the surface precipitation reaction of Heilbrunn. Actually, quantitative data exist (Mazia, 1937, pp. 299-300) which indicate that media low in calcium (potassium chloride and potassium citrate) are effective in causing an increase in free calcium in the *Arbacia* egg—presumably by releasing bound calcium from the cortex. The conclusion that the fertilized egg cortex consists, to some extent, of a masked calcium compound is inescapable.

The preceding paragraphs have been devoted to enumerating the evidences for ion release in a definite protoplasmic region of the living cell. This phenomenon offers, however, additional points of interest. Some of these we should like to mention briefly. In the first place, the phenomenon of "exploding" cells or cell granules is of considerable physiological importance. There is, of course, an extensive literature on exploding platelets in the field of mammalian blood-clotting. More important for our discussion, perhaps, are the observations of Donnellon (1938) on the nature of the clotting of the perivisceral fluid of the sea urchin. He found that the formation of the "cell clot" is accompanied by an explosion of the red granules of the "red amoebocytes" and of the "colorless" granules of the "white" ones. Moreover, there is a biochemical parallelism in that isotonic KCl is especially effective in causing granule breakdown.

Perhaps the most interesting feature of this work lies in the fact that it represents a clear case of a difference in the physiological behavior of the cell surface at different times after fertilization. For, whereas much attention has been devoted to demonstrating differences in the physiological properties of the cell before and after fertilization, comparatively little stress has been laid upon the fact that the properties of the cell surface are progressively changing following insemination. There are several indications in the literature that this is so. Thus, Kopac (1938), studying the phenomenon of coalescence of *Arbacia* eggs with oil drops, remarks: "The coalescencies of fertilized eggs

if kept in sea water decrease steadily and become zero after 15 or more minutes." With respect to water permeability, Lucké and McCutcheon (1932, p. 123) state:

Returning to fertilized *Arbacia* eggs it was found that increase in permeability is not instantaneous and is in greater part completed during the next 10 minutes. This was demonstrated by transferring eggs to hypotonic sea water at different intervals after insemination. During the first few minutes after sperm has been added, eggs in hypertonic solutions shrink slowly and remain round like fertilized eggs. After 5 to 6 minutes from the time of insemination, noticeable increase in rate of shrinking occurs and this becomes more rapid at the same time that the tendency to crenation becomes more pronounced. Increased water permeability appears to remain as a permanent quality of the developing egg and may readily be demonstrated in the 2-cell and 4-cell stages in intervals between cleavage.

It is therefore clear that, following insemination, the cell surface is changing and progressively approaching some sort of equilibrium which is attained about the time of monaster formation.

With respect to the permeability characteristics of the cell surface at this time, an interesting problem is encountered in attempting to explain the instantaneous égress of the pigment from the living cell. Dialysis experiments with collodion membranes indicate that the pigment may be of high molecular weight, since it does not dialyze over a considerable period of time.¹ (Lederer and Glaser, 1938, assign the following empirical formula to the echinochrome extracted from the ovaries of *Arbacia aequituberculata*: $C_{12}H_{10}O_7$.) The question arises as to the mechanism whereby this large molecule passes out of the living cell. Eggs immersed in the pigment do not take it up—i.e., the cell does not seem to be freely permeable to it. Furthermore, why does the pigment diffuse out of, rather than into, the interior of the egg? To these and other interesting questions raised during the course of this investigation we are at present unable to give an answer. We may remark, however, that the properties of the cell surface appear to find highly suggestive analogies in the systems of protein and fatty-acid films described by Langmuir, Schaeffer, and Wrinch (1937).

SUMMARY

The red-pigment granules of the *Arbacia* egg have been used as indicators of ionic changes in the cell cortex. It is known that these granules disintegrate in the presence of free calcium ions but remain intact in isotonic potassium solutions or when treated with oxalate or citrate. When unfertilized eggs, or fertilized eggs that have not reached the monaster stage, are immersed in 1:1 proportions of isotonic potassium chloride, potassium oxalate, or potassium citrate to sea water, nothing happens to the chromatophores. This, in accordance with the foregoing observations, was to be expected. However, when fertilized eggs, in which the red granules are localized at the cell cortex (monaster stage), are treated with these reagents, many of the red granules disintegrate and release their pigment to the exterior. The cells remain alive and intact; there is no cytolysis. We conclude (1) that after the monaster stage the cortex of the fertilized egg

¹ The appearance of a faint green color in the dialysate is an indication that the orange pigment released by the action of potassium may consist of a mixture of pigments. In this respect we quote from Glaser and Lederer (1939), who find that "dans *Arbacia*, l'échinochrome est accompagné d'un pigment plus foncé l'iso-échinochrome (fines aiguilles brun rouge; F. 247°; mieux adsorbé; soluble dans l'éther; bandes d'absorption à 548 et 510 mμ dans $CHCl_3$), et d'un deuxième pigment (insoluble dans l'éther, soluble dans l'alcool butylique; bandes d'absorption, 515 et 480 mμ dans l'acétate d'éthyle)."

contains as a component a masked calcium compound and (2) that potassium can release this bound calcium, which then causes granule breakdown. In addition, brief references have been made to various aspects of this phenomenon which may be of interest to the cellular physiologist. Especial stress has been laid on the possibility of analyzing the biochemical and physiological properties of the cell cortex by the procedures outlined.

We are very happy to thank Dr. L. V. Heilbrunn for his unfailing interest, encouragement, and ready counsel.

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PLATE I

Photographs of test tubes containing unfertilized and fertilized eggs at various stages of early development after treatment with an isotonic KCl solution (see text). The layer of cells can be seen at the bottom of each tube. The color of the supernatant fluid changes upon standing. Furthermore, the pigment assumes different colors in the presence of different ions. For a study of the color chemistry of such systems the reader is referred to a paper by Vlès, Gex, and Riess (1928). A detailed discussion of the conditions under which the living eggs release their pigment to the supernatant fluid is presented under "Observations."

PLATE II

Selected stages from photomicrographs (unretouched) of the breakdown of the red-pigment granules of a centrifuged, fertilized *Arbacia* egg when treated with an isotonic KCl solution (see text). The focus is upon the upper surface of the hyaline area, wherein the red chromatophores are imbedded. The large black "dots" are the red-pigment granules, and their successive disintegration can easily be traced. It is interesting to note that these "granules" are not uniform in shape or in size. The prints were made, as numbered, from motion-picture negatives taken at standard speed, 16 frames per second.

PLATE I

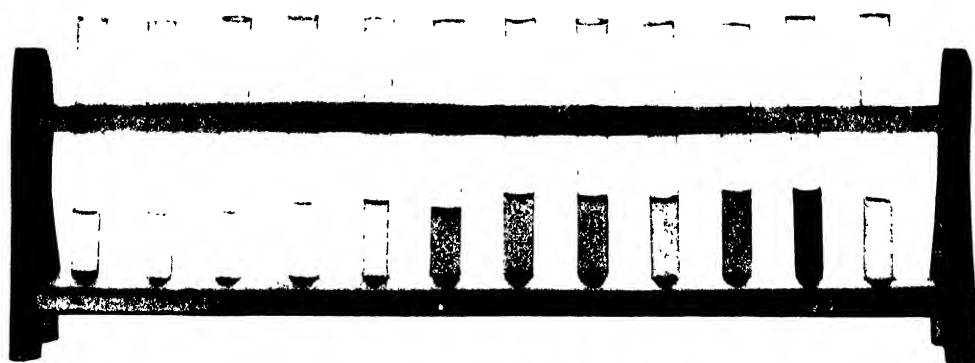


PLATE II

1



3



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9



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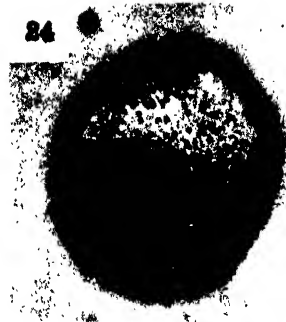
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33



THE EFFECT OF THERMAL INCREMENTS ON AND THE SUBSEQUENT ADJUSTMENT OF THE PROTOPLASMIC VISCOSITY OF AMOEBA PROTEUS

(Two figures)

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TEMPERATURE is one of the most thoroughly investigated variables affecting biological systems. While most investigations have been concerned with thermal increments which are relatively slowly applied, it has been impressed infrequently upon the investigator that thermal increments which are suddenly applied are to be interpreted, on the basis of experimental results, differently from those which are less rapidly applied. Thermal changes which are applied suddenly within the biokinetic range, and possibly those increments of a potentially lethal nature (e.g., "superoptimal") which are applied for very short periods of time, are to be considered as stimulative in character.

In the case of single cells Dutrochet and Becquerel (1837), working on the alga, *Chara fragilis*, were the first investigators to record the effect of suddenly applied thermal changes. Since this work, most of the investigations have been concerned with botanical forms in which suddenly applied thermal increments may cause a temporary cessation, retardation, or acceleration in the speed of protoplasmic streaming. The monograph of Ewart (1903) should be consulted for a review of this phase of the literature. In view of the present state of our knowledge and of Heilbronn's experiments (1914) it is inopportune for one to interpret cyclosis in terms of viscosity data and to attempt an appraisal of the literature on sudden thermal changes on cyclosis in the light of the present studies.

Little direct work has been undertaken with the view of suddenly applying thermal increments on animal cells with the exception, perhaps, of inducing artificial parthenogenesis in certain invertebrate eggs, though here, apparently, other factors are to be considered. A review of the literature in these fields may be obtained from chapter xi of Bělehrádek's book (1935). Recently Hopkins (1937) has studied the effect of adaptation to temperature in the locomotion-rate of the marine amoeba, *Flabellula mira*.

This investigation was originally planned to show the effect of sudden application of, and recovery from, heat when applied, within the biokinetic range, on the highly labile plasmagel of *Amoeba proteus*. The preliminary experiments were performed during the Thanksgiving recess (1936) in Dr. D. L. Hopkins' laboratory at Duke University. It was Dr. Hopkins' intention to correlate the rate of locomotion with viscosity data; but, as monopodal amoebae invariably underwent poly podality at some time during experimentation, measurements of the locomotion-rate obviously were futile.

¹ This investigation was aided by a grant from the Faculty Research Committee, University of Pennsylvania.

I wish to thank Professor C. E. McClung for his many kindnesses during the course of this work. I also wish to express my gratitude to him and to Dr. E. R. Helwig for their kind co-operation in arranging for me to use their much-needed temperature room.

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² I wish to thank Dr. D. L. Hopkins for the facilities placed at my disposal during my sojourn in his laboratory.

MATERIAL AND METHODS

The material employed in this investigation was *A. proteus*; the specific cytoplasmic layer studied was the plasmagel. Amoeba cultures were prepared from stocks originally purchased from the Carolina Biological Supply Company and from Professor A. A. Schaeffer. Amoebae were cultured in 4-inch finger bowls to which quartz distilled water was added until a depth of about 15 mm. was obtained. To each culture was added two or three sterile wheat grains plus a few drops of culture fluid containing *Chilomonas*. The cultures were then placed in a constant-temperature cabinet. After 3 or 4 weeks the wheat grains were removed, and the cultures were tested periodically for "normal" viscosity. The temperature cabinet was maintained at a fairly constant temperature at least 5 days prior to experimentation. The temperature did not fluctuate more than 3° C. during this period. Likewise the pH of the culture medium was maintained between 6.6 and 6.8.

The viscosity of the protoplasm was determined by the centrifuge method. An Emerson hand centrifuge, which developed a centrifugal force of 2,474 times gravity when the handle was turned at the rate of one revolution per 2 seconds, was employed in these experiments. The end-point used in comparing the viscosity of the experimentally treated with the control or "normal" amoebae was the time in seconds necessary for a constantly applied centrifugal force to move the intracellular crystals into one-half of the more or less spherical cell. The time in seconds necessary to achieve this end-point is known as the "centrifuging value."

The experiments were performed in the following manner. Amoebae were reared at a fairly constant temperature ± 1.5 C. and pH 6.6–6.8 for a 5-day period prior to experimentation. The initial temperature to be employed was determined from the nature of the experiment in question. All temperatures studied were within the biokinetic range for culturing amoebae. When any amoebae culture was ready for experimental use, the culture bowl, containing amoebae, was placed in a glass receptacle, the inner diameter of which was 7.5 inches; concentric with this was a larger glass vessel, the diameter of which was 9 inches. The space between the two concentric glass vessels was packed with thoroughly dried sawdust and sealed by a plastic cement. On the bottom surface there was an area of such dimensions as to permit transmitted light to illuminate the culture when under a large-field binocular microscope. This jacket served to prevent currents of air from exerting any sudden change of temperature on the undersurface of the culture bowl to which amoebae were generally attached. Thus, all surfaces of amoebae were exposed to approximately the same temperature, as indicated.

Upon removal from the constant-temperature cabinet the culture bowl containing amoebae was placed in water of the same temperature as that of the culture medium. When 80 per cent, or slightly more, of the amoebae showed a centrifuging value between 140 and 160, the amoebae were declared "normal" and were ready for experimental purposes. Amoebae were then removed to the thermostatically controlled temperature room. The procedure then followed either one of two methods according to the nature of the experiment in question.

a) *Slowly applied thermal increments, e.g., 11°6–26° C./ca. 200 minutes (Fig. 1).*—The culture bowl, immersed in the larger double-jacket glass vessel containing water at the same temperature as the cabinet, was placed in a constant-temperature room with thermometers immersed respectively in the culture and jacket fluids. The experiment was

timed from the moment the culture was brought into the higher-temperature room. Centrifuge tests were made consecutively, and the rate of increase of the temperature in jacket and culture fluid was recorded immediately prior to each centrifuging. The temperature differential between the two fluids was never greater than 1°C . Centrifuge tests were made in an anteroom, the temperature of which was initially comparable to that of the cabinet temperature and was permitted to increase synchronously with the thermal increment to which the amoeba culture was experimentally subjected. While the method did not permit perfect synchronization between anteroom and culture temperatures, it was possible to keep them within 3°C . (maximum) of each other. This differential between culture and centrifuging temperatures does not serve to stimulate the plasmagel.

*b) Suddenly applied thermal increments, e.g., 10°C . or more rise in temperature within 1-10 minutes (Fig. 2).—*The same procedure was followed as described under (a), only the temperature of the culture medium was increased to within 2° or 3° of room temperature by the addition of preheated culture medium which was never warmer than 30°C ., and thus below the lethal temperature for this species of *Amoeba* (see Thornton, 1932, 1935). The time was recorded from the initial application of the warmed, culture medium to the culture bowl. For slower application of temperature (e.g., curve B, Fig. 2) preheated culture fluid was added at such a rate that the desired temperature was attained progressively over a 10-minute period. In all cases the fluid in the various culture bowls was gently stirred as the warmed culture fluid was slowly added.

In developing a curve, eight to twelve amoebae were centrifuged at a value slightly higher than that anticipated on the bases of preliminary experiments. As fast as one could work, this point was investigated until the desired end-point was obtained. After every positive (end-point appearing in over 80 per cent and less than 100 per cent of the centrifuged amoebae) centrifuging value was obtained for those portions of the curves (Figs. 1 and 2) which show rapid change of value, a further centrifuge test was employed in order to determine that the positive value represented was succeeded, in turn, by one of negative character. Succeeding centrifuge values were determined in a comparable manner.

By employing the technique already described, the viscosity curves (Fig. 1) were investigated at six different times, while the 2-hour studies of the curves which represent over a 10°C . change within 1-10 minutes (Fig. 2, curves A and B) were performed seventeen times, and those curves showing recovery (Fig. 2, curve A') were studied five times. The recovery curves were always investigated immediately after the temperature had been increased to within 1° - 2°C . of the new thermal environment. All experiments represent different amoebae cultures, while curves A, B, and A' represent data as plotted from one stock culture.

RESULTS

*a) Slowly applied thermal increments, e.g., 11°C - 26°C ./ca. 200 minutes (Fig. 1).—*When one subjects *A. proteus* to a thermal increment as represented by the broken-line curve the co-ordinates of which represent the time-rate of thermal increase, it is found that there is a progressive decrease (solid-line curve) in the centrifuging value for each arbitrary progressive increase in temperature. The solid-line curve, representing data as obtained for one amoebae culture, shows the centrifuging value (viscosity) plotted as a function of the time of exposure in minutes to the thermal increment as expressed by

the broken-line curve. In the experiments thus recorded, starting with a culture temperature of 11.6°C . and a centrifuging value of 140 seconds, the temperature increases in 58 minutes to 18.6°C ., while the centrifuging value decreases simultaneously to 90 seconds, i.e., a 36 per cent decrease; after 156 minutes the temperature increases to 24.3°C . and the viscosity decreases simultaneously to a centrifuging value of 40 seconds. Finally the culture temperature increases and levels at 26°C .; the centrifuging value levels at 40 seconds. When the temperature is maintained at this value, the viscosity does not change within 8 hours after the beginning of the experiment, nor when a determination is made the following day (about 24 hours after the start of the experiment).

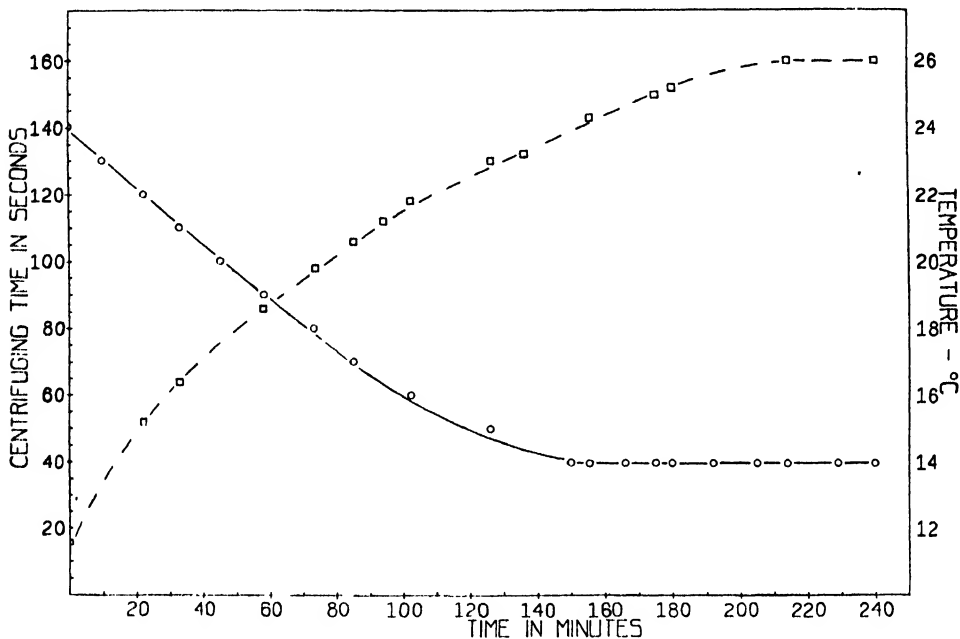


FIG. 1.—The solid-line curve (○) represents the centrifuging time in seconds (viscosity) of the plasmagel of *A. proteus* versus time in minutes during which the viscosity is subjected simultaneously to the thermal increment as represented by the broken-line (□) curve. The latter curve shows the time-rate of thermal increase to which the amoeba culture is subjected. pH, 6.6–6.8.

b) *Suddenly applied thermal increments, e.g., 10°C . or more rise in temperature within 1–10 minutes (Fig. 2).*—The data for two experiments are represented by the curves in which centrifuging values are plotted as functions of the time in minutes during which the viscosity of amoebae was investigated when the amoeba culture was subjected to thermal increments of 10° or more above room temperature within an interval of 1–10 minutes.

Curve A shows the results of one experiment in which the culture temperature was raised from 16°C . to a final value of 27°C . within 10 minutes. After a lapse of 11 minutes from incipient temperature increase, the centrifuging value decreases 37 per cent, while 24.5 minutes later there is a 75 per cent decrease from the “normal,” or control, viscosity value. Further lapse of time finds no apparent change in the centrifuging value

until after 38 minutes, when the viscosity progressively increases; and at 109 minutes the centrifuging value has gained 275 per cent over its previous minimum value.

Curve *B* (Fig. 2) represents another amoeba culture which was brought from a temperature of 12° C. to 26° C. within 1 minute. Thus the decrement in the centrifuging value is steeper (cf. curves *A* and *B*), so that after 3 minutes from the incipient thermal increase the viscosity has decreased 62 per cent, while after 9 minutes a decrease of 81 per cent is attained. Similarly, the viscosity levels at a transient minimum value which

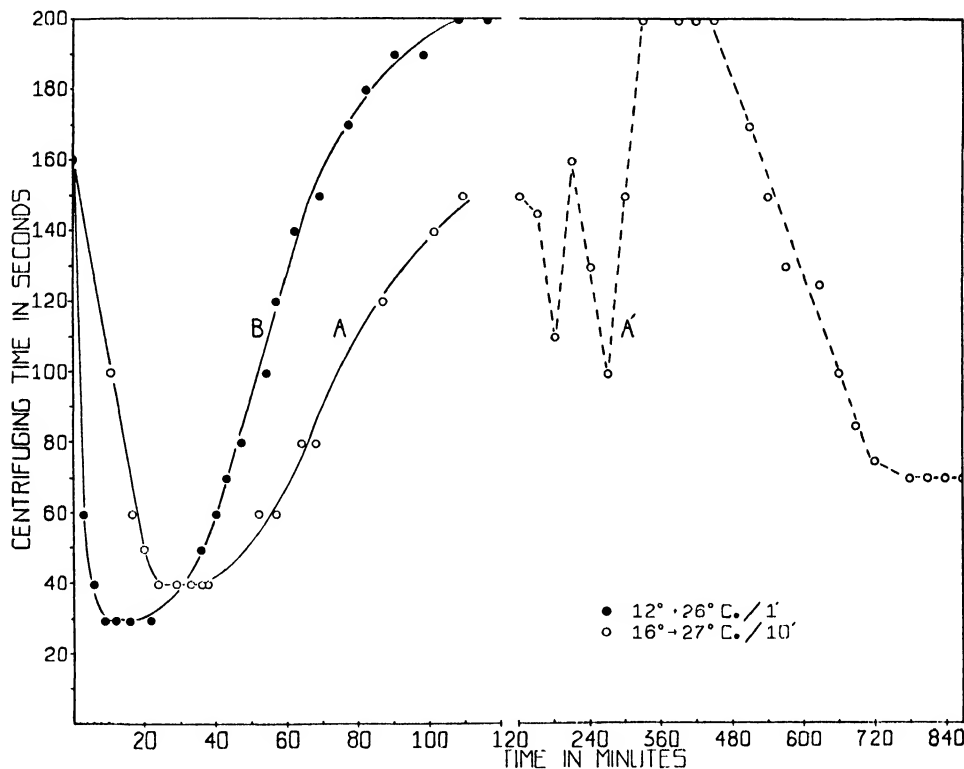


FIG. 2.—Centrifuging time in seconds (viscosity) of the plasmagel of *A. proteus* versus time in minutes from the initial exposure to the thermal increment thus indicated. Curve *A'* is a continuation of curve *A*. pH, 6.6–6.8.

is maintained for approximately the next 13 minutes, after which the centrifuging value progressively increases with time. After 116 minutes it has increased 566 per cent from the previous minimum value.

Curve *A'*, a continuation of curve *A* (Fig. 2), shows the fluctuations in the centrifuging value when a study is undertaken of the period of recovery from the application of a relatively steep thermal increment. These data were not obtained on a minute-to-minute basis, as in the 120-minute curves (represented by curves *A* and *B*, Fig. 2), but during 15–30-minute intervals after the termination of the initial 120-minute interval. It was recognized, even on the basis of preliminary experiments, that after the first minimum and

maximum all subsequent minima and maxima, representing the fluctuating centrifuging values, bear no constant relation to each other when different experiments, i.e., different amoeba cultures, are compared with respect to the time (abscissas) at which these values are respectively attained. The first minimum was always quantitatively reproducible within the limits of experimental error; the first maximum was predictable as regards the time variable (abscissas) but not with respect to the viscosity value (ordinates). Hereafter the fluctuations were predictable as such, but never from a quantitative viewpoint with regard to time or centrifuging value. Within $9\frac{1}{2}$ –14 hours the plasmagel assumed a constant centrifuging value, which was maintained the following day (approximately 10–12 hours after close of the experiment.)

In three experiments in which a thermal increment of shorter temperature range (5° C. increase above cabinet temperature) was applied within the shortest time possible (1 minute) under conditions of the technique here described, it was found that such an increment does not produce results comparable to those of greater thermal magnitude, i.e., an increase of 10° C. or more.

DISCUSSION

When the plasmagel of *A. proteus* is subjected to a relatively slow thermal increase, e.g., from 11.6° C. to 26° C. within approximately 200 minutes, there is a progressive decrease in the viscosity which more or less parallels synchronously the progressive increase in temperature to which the amoebae are exposed. It is common knowledge that most inanimate gels undergo a progressive liquefaction when subjected to a temperature increase. Similarly, in the majority of experiments on protoplasmic systems, Fauré-Fremiet (1913); Weber (1916), Pantin (1924), Costello (1934), and, for the temperature range as herein studied, Thornton (1935), working on the same species, find a progressive decrease in the viscosity as the temperature increases. In view of our limited knowledge as to the factor or factors involved in, and the criticism of Heilbronn (1914) with regard to, the relation of the speed of chloroplastid streaming to the viscosity of the protoplasmic system in question, it would be superfluous to attempt any analogy. A review of this field may be obtained from Ewart (1903) for the earlier, and Bělehrádek (1935) for the later, literature.

The apparent incongruity between the time at which the temperature and the centrifuging-value curves (Fig. 1) approach asymptotically their respective maximum and minimum ordinates may be ascribed at least partially to the lag between the successive temperature changes, as induced in the culture fluid, and their transmission to the protoplasm of amoebae. Perhaps the greater factor is that, as the temperature and viscosity respectively approach their constant or leveling values, they do so by increasingly smaller and smaller increments. Thus, considering the experimental error inherent in the centrifuge method, a difference as produced in the viscosity by 1° of temperature is impossible of direct measurement.

The primary reason for which these experiments were pursued was to study the behavior of the plasmagel to suddenly applied thermal increments when acting within the biokinetic range. Thermal increments that are more suddenly applied (Fig. 2) produce steeper decrements in viscosity, and the respective minimum centrifuging value attained is lower the more suddenly the thermal increment is applied, and, within the limits of the temperature range herein explored, is independent of the value to which the temperature is experimentally elevated. Although there is no literature apparently on this subject,

except the study of Hopkins (1937) on the adaptation of *F. mira* to temperature and that based on the chloroplastid streaming method, nevertheless there is an analogous behavior when one compares the action of various stimulating agents on the plasmagel of *A. proteus*. That this layer is a highly thixotropic gel may be concluded from the experiments of Angerer (1936); and that various stimulating agents will cause a transitory liquefaction of this layer is shown by the action of such stimulating agents as ultra-violet radiations (Heilbrunn and Daugherty, 1933), mechanical agitation, electric current (Angerer, 1936, 1937), and now for relatively steep thermal increments when applied within the biokinetic range. Heilbrunn (1937), on the basis of his and his students' work, has developed a colloid chemical theory of stimulation which anticipates such a reaction for the plasmagel.

Hopkins (1937) has shown that in the adaptation of the locomotion-rate in *F. mira* to temperature there is a progressive adjustment when the thermal increments are protracted while a long period of fluctuation ensues when thermal increments are more suddenly applied. If we assume that the physical state of the protoplasmic colloid is the sole factor in determining the rate of locomotion in the amoeboid cell (Pantin, 1923; Mast, 1926), Hopkins' results are explicable on the basis of the viscosity data as presented here.

The nature of the physical-chemical change or changes manifesting themselves during the series of viscosity fluctuations prior to the assumption of a constant value characteristic of the temperature in question is problematical at this time. According to the colloid chemical theory of stimulation as developed to date, calcium is the principal substance to be identified with the reaction induced by certain stimulating agents. Recently Heilbrunn and Daugherty (1938) have found that fat is released when *A. proteus* is subjected to ultra-violet radiations. On subjecting *A. proteus* to mechanical impacts, and again during the course of these experiments, there appeared an increase in the number of highly refractive "globules" within the cytoplasm. During the course of this work the "globules" were found to stain with Sudan III, and thus may be identified as fat. However, since all cells observed have contained some "globules" prior to experimental treatment, it is suggestive to treat of this material in at least a semiquantitative manner. These experiments are planned for the future. On the basis of the evidence available at this time, it would indicate that during the sudden breakdown of the plasmagel, calcium and possibly fat, among other substances, are temporarily liberated when various stimulating agents are employed.

In considering Thornton's (1932, 1935) technique for a study of the temperature-viscosity curve for *A. proteus*, it was found that tap water (19° C.), when placed in the same type of centrifuge tube which Thornton employed in subjecting amoebae to the higher temperatures of the temperature bath, attained the temperature of the water bath in less than 1 minute for temperatures up to 35° C. Thus, it would seem that that part of the temperature-viscosity curve which embraces the higher temperature range of his curve should be treated with reservation.

Likewise, values for temperature characteristics (μ) and temperature coefficients (Q_{10}) based on a technique which induces fluctuations in the viscosity values as represented (Fig. 2) can have, obviously, little meaning. This same viewpoint is emphasized by Hopkins (1937) in considering the adaptation in locomotion to temperature by *F. mira*.

SUMMARY

1. The viscosity of the plasmagel of *A. proteus*, when subjected to various thermal increments, was determined by the centrifuge method.

2. Slowly applied thermal increments (e.g., 11.6–26° C./ca. 200 minutes) produce simultaneously a correspondingly slow decrease in viscosity which levels at a value characteristic of the final temperature to which the amoeba culture is elevated.

3. On subjecting amoeba cultures to relatively steep thermal increments as applied here, there is produced a sudden decrement in viscosity which progressively decreases to a minimum value. The steeper the applied thermal increment employed, within the temperature range studied, the sharper the initial decrement and the lower the viscosity value attained.

4. When studying the recovery of the viscosity to an equilibrium value characteristic of the temperature in question, it is found that the viscosity passes through a succession of minima and maxima values. After the first minimum and maximum values are attained, there is quantitatively no predictable course through which the viscosity will pass, though 9½–14 hours are required before the viscosity of the plasmagel comes to a steady state.

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EXPERIMENTS WITH COLPIDIUM CAMPYLOIDES IN HIGH-FREQUENCY ELECTRIC AND MAGNETIC FIELDS

(8 figures)

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ONE has only to review the rapidly growing literature on the subject of radiotherapy to be impressed by the effectiveness of this form of treatment for a variety of human ills. However, from both laboratory and clinic have come several controversial questions relative to the nature and specificity of the action on tissues and organisms at different frequencies. As enumerated by Mortimer and Osborne (1935), the most important questions pertain to (1) degree and uniformity of penetration, (2) special selective thermal action, (3) specific biologic action, and (4) specific bactericidal action.

Of immediate interest to us is the problem of biological or bactericidal specificity upon which there is no unanimity of opinion at present. Haase and Schliephake (1931), Pflomm (1931), Liebesny (1932), Schliephake (1932), Stieböck (1933), Dausset and Dognon (1934), Jellinek (1936), and many others maintain that there are special effects of high-frequency fields which cannot be explained in terms of heat action alone. The other side of the picture is represented by an even longer list of competent observers who are unable to confirm heatless bactericidal action or any other specific electrical effect at high frequencies. With reference to the action of a 1-10 megacycles (Mc) electric field, Kovacs (1936) sums up this viewpoint: "Its primary physical effect is that of heat generation and this explains adequately its action" (p. 433).

We have approached this problem by preventing appreciable temperature variations during the exposure of individual protozoan organisms in minute quantities of media and by using the mean daily division-rate, as determined by the isolation culture method, as the criterion of general physiological condition after treatment. The data from these experiments comprise the substance of this report.

The work was done in the department of anatomy, Cornell University Medical College, New York City. We are greatly indebted to Professor J. B. Russell, of the electrical engineering department, Columbia University, for the use of two General Radio wave-meters, and to Mr. R. H. Hall, also of Columbia University, for the stock cultures of *Colpidium campyloides*. It is also a pleasure to acknowledge the helpful suggestions made by Mr. Dale Pollack, of the R.C.A. Manufacturing Company; Dr. Myron A. Coler, of the Paragon Paint and Varnish Corporation; and Mr. W. W. van Roosbroeck, of Bell Telephone Laboratories.

PLAN OF STUDY, MATERIAL, METHODS

Colpidium campyloides was selected for this work because it is an easily cultivated free-living type, small enough to become adjusted readily to temperature variations in the medium. It divides rapidly in drop cultures, and its structural and cyclical features are known fairly well (Sonneborn, 1932; Kidder and Diller, 1934).

Considerable attention was given to the maintenance of uniform environmental conditions throughout all phases of the work—salt concentration in the medium, temperature (25° C.), pH (5.1–5.3), and amount of inoculum transferred from culture to culture. The medium consisted of a rice-salt combination, R. H. Hall's modification (personal communication) of the formula given by Rohdenburg and Nagy (1937): KH_2PO_4 , 0.5 gm.; KNO_3 , 0.4 gm.; NaCl , 0.1 gm.; MgSO_4 , 0.1 gm.; rice, 5.0 gm.; water, 1,000 cc. The mixture was boiled for 10 minutes, filtered through cotton, and allowed to stand in an open vessel for 24 hours before use. In order to standardize the procedure, a stock strain of *Colpidium* was established in isolation culture (five lines) and carried for 90 days before the first experimental lines were taken from it.

An important part of the procedure was the method of preparing individual colpidia for exposure in the high-frequency fields. One organism within a minute quantity of medium was centered in a thin-walled capillary, 4 cm. in length, with the aid of a micro-injector. The ends were then sealed by flaming. Individual organisms encapsulated in this fashion were exposed for a standard time of 40 minutes. In all of the electric-field exposures, the capillaries were arranged in the center of the field, with long axes perpendicular to the field lines. For those runs in which thermal changes were to be avoided as far as possible, the capillaries were placed in the middle of a glass water jacket (Fig. 2), and the assembly lowered into the field. The control organisms were sealed under the same conditions but were placed in a 25° C. incubator for the 40-minute interval.

At each frequency setting, a number of encapsulated colpidia, all descendants of the single individual isolated 12 hours before, were exposed with water- and air-cooling. Twelve or more capillaries were prepared in the prescribed manner and divided equally into three groups. One group of at least 3 capillaries served as controls. The second group was treated for 40 minutes while in the water jacket. The jacket was then removed from the field, the frequency checked, and the third group exposed for a corresponding period without the water bath. The latter will be described as air-cooled exposures.

The capillaries were rinsed in alcohol, dried, and broken into fresh drops of medium for a 12-hour incubation period. Fission sometimes occurred within the capillary. In this event only one of the progeny was cultured. At the end of a 12-hour period there were usually between 10 and 32 organisms per drop. Five of these were isolated and continued in isolation cultures for at least 13 days. Thus, each experimental organism was propagated as a "series" of five lines. Although the number of divisions per day per series was computed on a 24-hour basis, we were obliged to isolate and to transfer this rapidly reproducing species at 12-hour intervals for the sake of accuracy in counting. At the forty-eighth hour all but one or two series from each of the three original groups were discontinued in order to make way for new series set up on successive days. Those continued were selected by serial numbers only. A total of 129 colpidia were treated in the high-frequency field during this investigation. Only 5 losses occurred, 3 of which could be attributed definitely to extraneous causes.

In another group of experiments, we were able to test the reactivity of encapsulated colpidia when subjected to either a homogeneous magnetic field or an inhomogeneous electric field at frequencies corresponding to those selected for the experiments already described. The possibility of an effect from a highly inhomogeneous field was suggested by the work of Kimball (1938), who reported a noticeable inhibition in the formation of yeast buds in a heterogeneous magnetic field, an effect that did not occur in a homo-

geneous field three thousand times more intense. Further, in a majority of practical applications, the radiotherapy field is not homogeneous. In our experiments only the electric field was made inhomogeneous.

We did not attempt to make exposures at frequencies higher than 60 Mc as this was the practical limit of the oscillator.

APPARATUS

Figure 1 is a schematic diagram of the apparatus designed to produce as pure a wave form as possible. The oscillator was a push-pull type in which all even-order harmonics

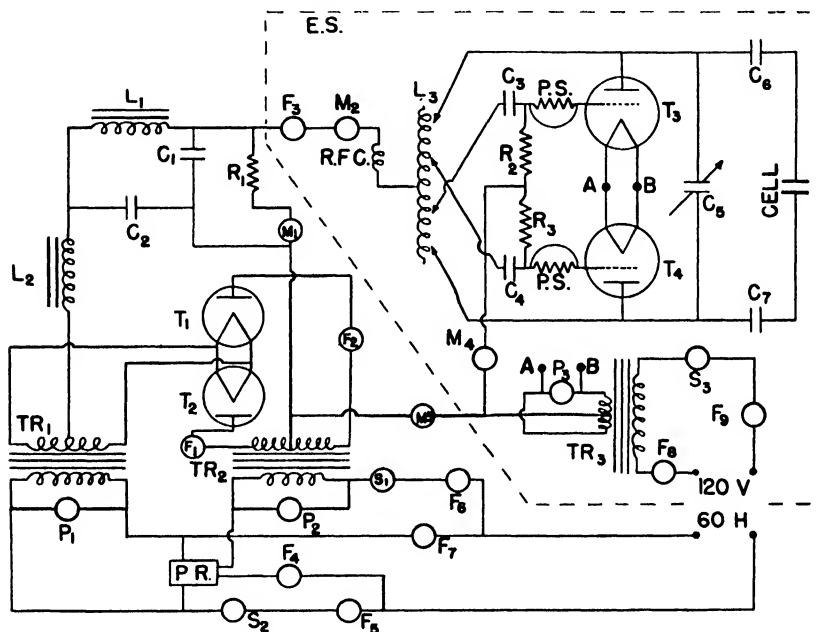


FIG. 1.—Schematic diagram of oscillator and rectifier. *E.S.*=electrostatic shield surrounding oscillator, *F*=fuse, *H*=hertz (cycles per second), *M*=meter, *P*=pilot, *P.R.*=protective relay, *P.S.*=parasite suppressor, *R.F.C.*=radio frequency choke, *S*=switch. *T*₁, *T*₂=type 866-A rectifier tubes, *T*₃, *T*₄=type 808 triode oscillator tubes. A Variac (not shown) in the primary of *TR*₃ permitted adjustment of the rectified voltage from 0 to 1500.

cancel when properly balanced. The *L-C* ratio was low (except at 60 Mc), which also reduced the harmonic content.

The water jacket and associated cooling apparatus is shown in Figure 2. The temperature at the outlet (*T*₂) did not exceed that at the inlet (*T*₁, approximately 25° C.) by more than 0.5° C. in any of the experiments.

The heterogeneous electric field was obtained by substituting for the two circular plates of the cell (Fig. 2) a lead zone-of-one base (diameter of base=35 mm.) and a tapered rod terminating in a sharp point at the zone's center of curvature (*r*=24 mm.). Two capillaries at a time were suspended perpendicular to the field axis, 10 mm. from the pointed electrode, and exposed for 40 minutes. The capillaries were prepared with

especially small quantities of medium in order that the swimming movements of the inclosed organisms would be limited to the central part of the field.

Another set of two to four capillaries was placed within a 2-inch length of ordinary 4-mm. glass tubing, and the whole was suspended in the center of the air-core inductance (L_3 , Fig. 1) by means of a silk thread. This method of utilizing the flux within the tank coil provided a magnetic field essentially like that set up in the inductothermic apparatus described by Merriman, Holmquest, and Osborne (1934) and by Kling (1936), where an extrinsic applicator coil was used instead of the conventional condenser plates. For our purpose it was of advantage to employ both electric and magnetic fields at the same time on different organisms.

Between 12 and 27 Mc (25–11 m.), we measured frequency with a G.R. Model 358 wavemeter and in the neighborhood of 60 Mc (5 m.), with a G.R. Model 458 wavemeter.

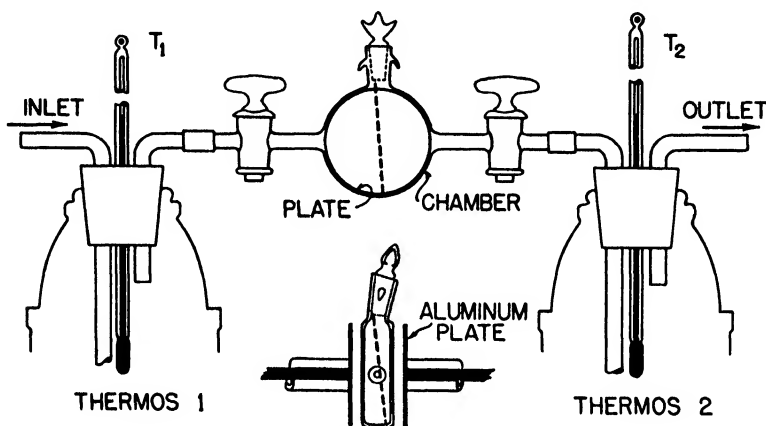


FIG. 2.—Diagram of water jacket and connections. The position of the capillary is indicated by the broken line. The lower center figure shows the relative positions of the condenser plates, water jacket, and capillary. The colpidia were confined to the mid-portion of the capillaries.

Neither of these responded in the intermediate range. To set the oscillator at 30 Mc (10 m.), we constructed an absorption wavemeter consisting of a small coil in series with a midget condenser and a $1/25$ -watt neon bulb mounted on Micalox and calibrated it against a G.R. Model 605-A standard-signal generator. This was done in the Marcellus Hartley Laboratory of Columbia University.

FIELD STRENGTH AT THE ORGANISM

There is considerable confusion in the literature of radiotherapy on the specification of dosage. This was initiated when early designers rated their machines in watts. The difficulty with the watt-minute as a unit of dosage is that it is impossible to know the wattage output of an oscillator without involved measurement. This cannot be done by the manufacturer and supplied as a part of the calibration of the machine because the output coupling differs with every movement of the wires or plates and with the treated material. Furthermore, an appreciable fraction of the power is often in harmonics and parasites, particularly in machines using raw alternating current on the anodes. We have used a different measure of the field at the organism—the volt per centimeter.

This is the practical unit of electric field strength or force on a unit charge. A satisfactory unit of dosage is then the volt-minute per centimeter.

The corresponding magnetic unit is the gauss-minute. The space average value of the magnetic field strength within a magnetized medium is equal to the quantity symbolized by B and called the "magnetic flux density" (Frisch, von Halban, and Koch, 1938).

It is the purpose of this section to evaluate in practical terms the strength of the field at the organism in each of the experiments. The symbols employed in the derivations are defined as follows:

A, C, P, Q	constants	l	see Figs. 3 and 4
a, b	see Fig. 3	N_n	Neumann function, order n
B	flux density, gauss	q	charge, esu
c	$3 \cdot 10^{10}$ cm. per second	r	radius, centimeters
E	electric field strength, esu	s	perpendicular (senkrecht)
e	empty (subscript)	u	velocity, centimeters per second
F	force, dynes	V	potential, esu
f	frequency, hertz (cps)	x, y	Cartesian co-ordinates
I	current, amperes	a	$K\omega^2 - 4\pi\gamma j\omega$
J_n	Bessel function, order n	γ	conductivity, esu
j	$\sqrt{-1}$	μ	permeability
K	dielectric coefficient	ϕ	see Fig. 3
		ω	periodicity ($2\pi f$)

The total force on a single charge in both electric and magnetic fields is

$$F = q \left(E + \frac{1}{c} u \times B \right). \quad (1)$$

The quantities E and B of equation (1) are to be evaluated for the particular case of a capillary within a high-frequency field.

a) *Electric field inside air-cooled capillary.*—In 1864 Maxwell showed that most, if not all, macroscopic problems in electricity are determined by applying the proper boundary conditions to the general solution of four vector-differential equations. Assuming all media in the field (Fig. 3) to be homogeneous and isotropic, we obtain from Maxwell's equations:

$$\nabla^2 E + \frac{\mu}{c^2} (K\omega^2 - 4\pi\gamma j\omega) E - \nabla \nabla \cdot E = 0. \quad (2)$$

The last term—namely, the gradient of the divergence of E —we set equal to zero, since it is assumed that no free charge is present anywhere in the field. The general solution is

$$E_{x1} = \sum_{n=0}^{\infty} (A_{n\pi 1} \cos n\phi + C_{n\pi 1} \sin n\phi) [P_{n\pi 1} J_n(a, r) + Q_{n\pi 1} N_n(a, r)], \quad (3)$$

with similar expressions for E_{y1} , E_{x2} , E_{y2} , E_{x3} , and E_{y3} .

While considerations of symmetry simplify the application of the boundary conditions, the exact solution is very involved. Fortunately, approximate methods lead to a

solution sufficiently exact for our purposes. The contribution of the imaginary term in equation (2) is negligible, and the relaxation period of any region is very small when

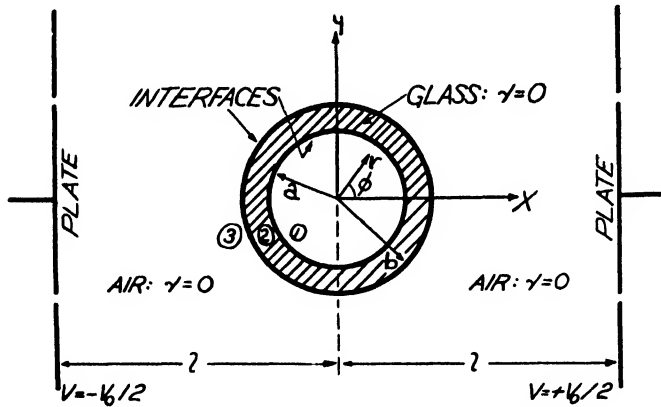


FIG. 3. - Schematized cross-section of an air-cooled capillary within the electric field

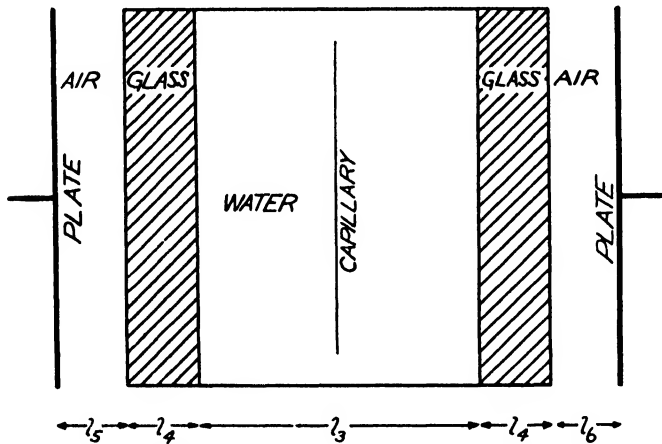


FIG. 4. - Schematized vertical section through water jacket with capillary in position

compared with the period of the oscillator. Therefore, we simplify equation (2) to the form:

$$\nabla^2 V = 0. \quad (4)$$

The boundary conditions are: (1) $V_1 = 0$ at $r = 0$, (2) $V_3 = -E_e r \cos \phi$ at large r , (3) $V_1 = V_2$ and $K_1 E_{r1} = K_2 E_{r2}$ at $r = a$, and (4) $V_2 = V_3$ and $K_2 E_{r2} = K_3 E_{r3}$ at $r = b$. The exact solution of equation (4) shows that the field within the capillary is uniform, in the x -direction (i.e., $E_{\theta 1} = 0$), and, when $l \gg a$, is equal to

$$E_{r1} = \frac{4K_2 K_3 E_e}{\frac{a^4}{b^2} (K_1 - K_2)(K_2 - K_3) + (K_1 + K_2)(K_2 + K_3)}. \quad (5)$$

TABLE 1

COMPARISON OF MEAN DAILY DIVISION-RATES IN TREATED AND CONTROL SERIES*

Frequency	13.0 Mc (23.1 m.)	19.1 Mc (15.7 m.)	30.0 Mc (10.0 m.)	57.2-58.8 Mc (5.1-5.25 m.)
Wave Length				
Stock series	6.8±0.21
Control	6.2±0.23
Difference	0.6±0.31
Percentage difference . .	9.7
R series	6.4±0.24	7.0±0.23	8.0±0.16	6.2±0.13
Control	6.2±0.23	7.5±0.30	7.9±0.16	6.7±0.12
Difference	0.2±0.33	-0.5±0.38	0.1±0.23	-0.5±0.18
Percentage difference . .	3.2	-6.6	1.3	-7.5
Field intensity	12-13	14-16	9.3-11	10-11
WR series	5.8±0.30†	7.2±0.23	7.9±0.20	6.1±0.17
Control	6.4±0.91†	7.5±0.28	8.0±0.16	6.7±0.12
Difference	-0.6±0.96	-0.3±0.36	-0.1±0.26	-0.6±0.22
Percentage difference . .	-9.4	-4.0	-1.3	-8.9
Field intensity	28	32	31
WR-A series	6.8±0.25	7.8±0.17	6.5±0.12
Control	7.4±0.27	8.1±0.09	6.7±0.12
Difference	-0.6±0.37	-0.3±0.19	-0.2±0.17
Percentage difference	-8.1	-3.7	-3.0
Field intensity	36	36	29
WR-B series	6.8±0.26	7.4±0.13	6.5±0.19
Control	7.8±0.16	8.1±0.09	6.7±0.12
Difference	-1.0±0.31	-0.7±0.16	-0.2±0.24
Percentage difference	-12.8	-8.6	-3.0
Field intensity	35	31	30
WR-C series	6.7±0.22	7.8±0.19	6.7±0.13
Control	7.7±0.18	8.0±0.13	6.9±0.13
Difference	-1.0±0.28	-0.2±0.23	-0.2±0.18
Percentage difference	-13.0	-2.5	-3.0
Field intensity	41	30	30

* The negative differences indicate that the division-rates in exposed series were less than the controls. The field intensity varied for each of the two or three water-cooled series (R) in each group; only the extremes are listed. Field intensity in peak volts per centimeter

† Mean determined for 6 days only (see Fig. 5).

b) *Electric field inside water-cooled capillary.*—Neglecting edge-effects, the field in region n of the homogeneous, isotropic system of Figure 4 is uniform and equal to

$$E_n = \frac{V}{\sum_{i=1}^n l_i \frac{K_n}{K_i}}. \quad (6)$$

Combining equations (5) and (6), the field within a capillary placed in a uniform water jacket is

$$E_{21} = \frac{4K_2K_3V}{\left[\frac{a^2}{b^2} (K_1 - K_2)(K_2 - K_3) + (K_1 + K_2)(K_2 + K_3) \right] \left[l_3 + 2l_4 \frac{K_3}{K_4} + 2l_5 \frac{K_3}{K_5} \right]}, \quad (7)$$

where l_3 is taken equal to l_6 .

c) *Magnetic field inside air-cooled capillary.*—The magnetic field strength between two plates connected to an oscillator is not appreciable below a frequency of about 3,000

TABLE 2

THE TOTAL NUMBER OF GENERATIONS PRODUCED IN 48 HOURS BY COLPIDIA EXPOSED TO MAGNETIC AND INHOMOGENEOUS ELECTRIC FIELDS*

		FREQUENCY			
		15 Mc	20 Mc	30 Mc	60 Mc
Control	First series	10.8	16.2	16.0	14.2
	Second series	9.6	14.8	15.8	15.2
	Mean	10.2	15.5	15.9	14.7
Inhomogeneous electric field	First series	9.6	16.0	14.6	18.6
	Second series	10.2	14.0	16.0	17.0
	Mean	9.9	15.0	15.3	17.8
Homogeneous magnetic field	First series	10.4	16.2	16.6	16.4
	Second series	10.4	15.8	14.8	15.0
	Mean	10.4	16.0	15.7	15.7
	Field intensity	5.7 gauss	3.2 gauss	1.9 gauss

* Magnetic field intensity in peak gauss.

Mc (Mason and Weaver, 1929). The highest frequency in these experiments was 60 Mc. At the center of a coil, the field strength is approximately

$$B = \frac{1}{10} \pi \mu NI, \quad (8)$$

where here N means the number of turns per centimeter.

d) *Inhomogeneous electric field.*—In order to make dE/dx as large as possible, we made the electrode point very sharp, thereby sacrificing our ability to calculate it.

e) *Numerical values.*—The dielectric coefficient of pyrex glass is 4.8. Goldsmith (1937) and Knerr (1937) have shown that the index of refraction of water (n) is still about 9, even at a wave length of 24 cm. Therefore we take the dielectric coefficient of water (K) as 81, the same as its direct-current value, since $n^2 = \mu K$. The permeability (μ) of water and glass is 1. The dielectric coefficient of dilute electrolytes has been measured at high frequencies (Holzer and Weissenberg, 1935) and found to be the same as water.

No anomalous dispersion is to be expected at the frequencies employed. The peak radio-frequency voltage across the tank of an unloaded push-pull oscillator is approximately twice the value of the applied direct-current voltage. We measured the circulating current in the tank coil (except at 60 Mc) with a radio-frequency ammeter. By putting these data and the physical dimensions into equations (5), (7), and (8), we obtain the values listed in Tables 1 and 2.

A typical capillary had an outside diameter of $150\ \mu$, a wall of $27\ \mu$, and was filled with medium for 15 mm. of its length.

RESULTS

a) *Homogeneous electric field*.—The culture records from the homogeneous electric field experiments are summarized graphically in Figures 5-8, inclusive. The ordinates represent divisions per day, while the abscissas indicate successive days. Each curve

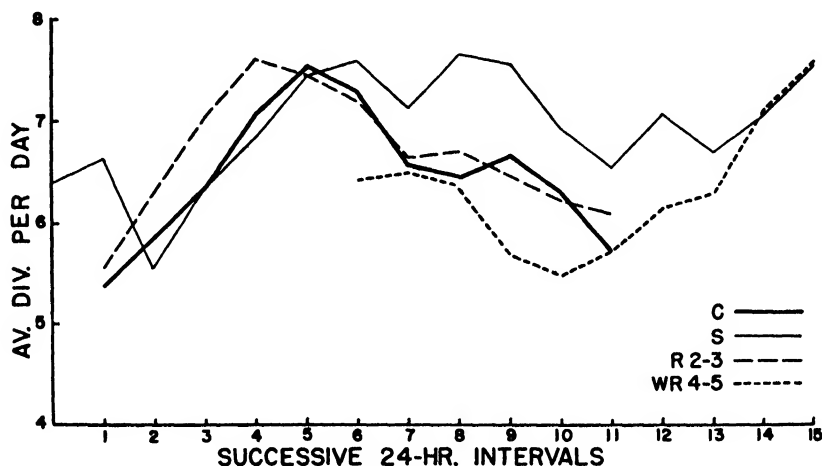


FIG. 5.—Culture records of colpidia exposed to 13.0 Mc homogeneous electric fields. Curves C and S represent one series each of control and stock cultures. Two series each of water-cooled (R 2-3) and air-cooled (WR 4-5) exposures are shown.

represents a 3-day moving average of the number of divisions in 24 hours for one or more sister series, i.e., series established from sister organisms treated under identical conditions. To illustrate: the curve WR 15-16-17 (Fig. 6) is the record of three air-cooled series; each point on the curve therefore gives the mean number of divisions for fifteen individual cultures taken 3 days at a time. It should be noticed that only a magnified portion of the ordinate axis is shown in each figure.

The division-rates of the "stock" series (S), one control series (C), and the experimental series treated at 13.0 Mc are compared for a period of 11 days in Figure 5. The organisms comprising the control and water-cooled series (R 2-3) were taken from the same stock culture. The air-cooled series (WR 4-5) were derived from the control strain on the sixth day.

The division-rates of organisms exposed at a frequency of 19.1 Mc are illustrated in Figure 6. One control series (C) and the water-cooled series (R 12-13-14) were estab-

lished from the stock series whose record is not included in the figure. At the end of the first 24-hour period, a group of colpidia were isolated and treated without the water bath. Three sister series (*WR 15-16-17*) were established from this group.

Exposures were also made to determine whether there are cumulative effects of successive treatments. At the end of the first 24 hours, colpidia from series *WR 15* were given a 40-minute air-cooled exposure. Curve *WR 15-A* is the record of one series set up in this manner. Similarly, *WR 15-B* was derived from *WR 15-A*, and *WR 15-C* from *WR 15-B* at successive 24-hour intervals. Thus, on the sixth day of the experiment shown in Figure 6, there were ten series, a total of fifty cultures. This number of cultures represents but one-third of the organisms actually exposed at this frequency.

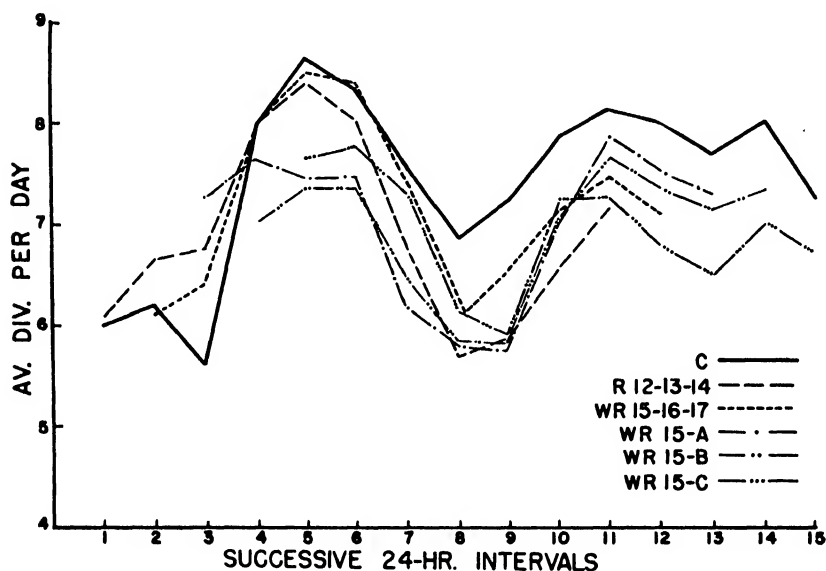


FIG. 6.— Culture records of colpidia exposed to 19.1 Mc homogeneous electric fields. One control series (*C*) and three series each from water-cooled (*R 12-13-14*) and air-cooled (*WR 15-16-17*) exposures are plotted. Curves *WR 15-A*, *B*, and *C* are single series of successively exposed organisms.

Twenty-two other exposed individuals were carried only to the forty-eighth hour. Their 48-hour records agree favorably with those shown in the figure.

Figures 7 and 8 are culture records of individuals exposed at 30.0 and 57.2 Mc, respectively. In order to attain the latter frequency, it was necessary to eliminate the tuning condenser (*C*, Fig. 1) and to make all adjustments by shifting the taps on the tank coil (*L*, Fig. 1). In view of the task of balancing the oscillator in this manner, it was not retuned after the removal of the water jacket. Accordingly, the water-cooled series in Figure 8 were exposed at 57.2 Mc, and the air-cooled series at 58.8 Mc.

The superimposed companion curves in each figure reveal a decided tendency to shift in comparable directions from day to day. In the six curves of Figure 6, for example, there are two maxima—one on the fifth and a second on the eleventh day. It is our belief that this conformity indicates uniform environmental conditions on a given day

and, further, that the differences in division-rate from day to day are due to variations in the bacterial content of the 24-hour medium. It was hardly feasible to attempt bacterial control in conjunction with the isolation-culture method. However, as long as the haphazard method of seeding the freshly made medium provided a favorable environment, its precise bacterial content was of no great consequence, since all cultures running on the same day were made up from the same batch of medium.

Interestingly enough, it is apparent that, with few exceptions (e.g., *WR 25-B*, Fig. 7), the curves of the water-cooled and air-cooled series are closely related and do not vary consistently in one direction from each other. For reasons to be considered in a later section, the temperature rise in the air-cooled capillaries was not sufficient to impair the subsequent reproductive capacities of the inclosed organisms.

More to the point, however, is the reproductive behavior of the exposed organisms in relation to the controls. If due regard is given to the magnified ordinates in the line

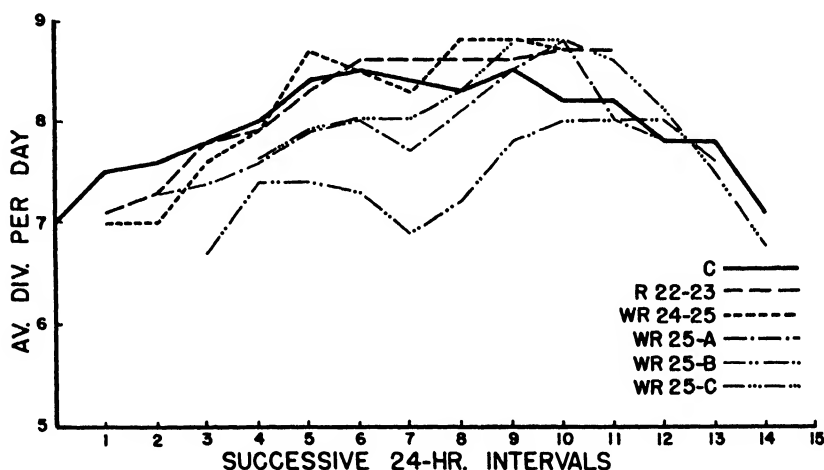


FIG. 7.—Culture records of colpidia treated with 30.0 Mc homogeneous electric fields. Curve *C* represents two control series.

graphs, the differences between the curves of experimental animals and the controls for corresponding periods are comparatively small. Although the magnitude of the differences may be of no consequence, the consistently diminished fission-rate in most of the exposed series introduces an element of doubt which must be taken into consideration.

An objective evaluation of these differences may be obtained from Table 1. Each value represents the mean number of divisions per day, together with its probable error, for 13-day culture records of from one to three series. The computations were made directly from the protocols, without regard to moving averages. The values of the controls are given for corresponding periods. Of the seventeen exposed groups, the mean rate of division exceeds the controls in two cases. In seven cases it is 0-5 per cent less than the controls, six are 5-10 per cent below, and two are 12-13 per cent below the controls. On purely statistical grounds we are unable to urge that the differences are significant. The differences between the values for treated and control organisms in Table 1 are three times greater than the probable error of the differences in only three of the seven-

teen cases. However, the confidence with which we regard the differences as of no consequence rests upon supplementary evidence.

The differences are not proportional to the length of exposure in the successively treated cultures. This is illustrated in Figures 7-8, where the *WR-C* series show an almost consistently higher division-rate than their two immediate predecessors (*WR-A*, *WR-B*).

Furthermore, three colpidia were taken from the 24-hour *WR-C* series in each experiment, sealed into capillaries, and exposed continuously for an additional 6 hours. For 48 hours afterward, in isolation cultures, they appeared to be as vigorous as the parent strains. Perhaps even more indicative is the fact that they averaged approximately two divisions each while under the influence of the high-frequency field. As a further test of increased dosage, the strength of the field at 30.0 Mc was increased by a factor of 5 (to approximately 150 volts per centimeter). This was done by reducing the distance between

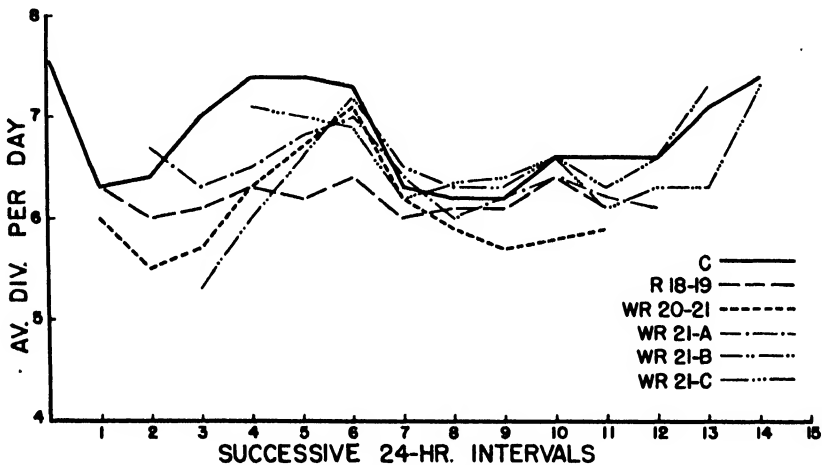


FIG. 8.— Culture records of colpidia exposed to 57.2–58.8 Mc homogeneous electric fields. Two series constitute the control curve (C).

the plates from 20 mm. to 4 mm. Three capillaries made up from *WR 25-C* on the twenty-fourth hour were suspended between the plates and given a 40-minute exposure. The subsequent 48 hours in isolation culture revealed nothing of interest.

The method of starting cultures from encapsulated colpidia presented a difficulty which was not completely overcome in this first group of experiments. The bacterial accumulations in isolation cultures are periodically sampled and passed on to new cultures at each transfer. The customary transfer pipette carries over to the new culture enough inoculum from the mother culture to preserve the sequence of bacteria. But, according to our procedure for preparing capillaries, the sealed-in droplet contained mother-culture fluid and fresh medium in the ratio of approximately 1 to 20. Moreover, the total volume of the dilute mixture actually seeded into the new drop culture by means of a capillary fragment was very small in comparison with that which is normally passed on in the pipette transfer.

Evidently this dilution factor was of some significance in depressing the division-rate

of many of the series, controls included, for the first few 24-hour intervals (Figs. 5-8, inclusive). Well-established isolation cultures of *Colpidium* were generally cloudy. The organisms were rounded and darkly colored. On the other hand, the cultures freshly seeded from capillary fragments were usually clear, and the colpidia slender and almost colorless for the first three or four transfers.

No attempt was made to compensate for this dilution factor when single treatments were given, for both control and exposed cultures were inoculated in the same manner. Whether or not the procedure varied sufficiently to account for the fact that single exposure series (*R* and *WR*) were preponderantly lower than the controls is conjectural. It is certain, however, that cultures established from the successively treated colpidia were affected. Each time, the new culture received a more dilute sample of mother-culture fluid taken from cultures established only 24 hours earlier and transferred at the first 12-hour interval. The condition was offset to some extent by fortifying each new culture with a pipette drop taken directly from the parent culture. In later experiments (Table 2), all the cultures were started with a supplementary drop from some other well-established culture. The results were more gratifying than before, in that the division-rate in exposed series was not predominantly lower than that of the controls.

b) *Heterogeneous electric and homogeneous magnetic fields*.—In order to explore further the possibility of affecting *Colpidium* within the selected range of frequencies, a limited number of organisms were exposed to inhomogeneous electric and homogeneous magnetic fields. The control and experimental animals were propagated in isolation cultures for only 48 hours after liberation from the capillaries. Beyond this point they were allowed to develop as mass cultures. Because of the few experiments which we were able to make, the results are indicative rather than conclusive.

Table 2 gives the total number of generations produced in 48 hours by individual series, each the mean of five lines. For the most part, the number of generations produced in 2 days by those treated at each frequency are in close agreement with the controls. Those exposed in the inhomogeneous electric field at 60 Mc, however, show a suspiciously increased division-rate. In the absence of further data from isolation cultures, it is necessary to rely upon records from the mass cultures for additional information. These records indicate that the observed differences at the forty-eighth hour are probably of little significance.

DISCUSSION

There appear to be two modes of energy transfer by which a high-frequency field may affect tissues or organisms. The first is that of heating the protoplasm, the medium, or both in the manner described by McClennan and Burton (1930). According to this method, different biological structures may be affected differentially, depending upon three factors: frequency, dielectric coefficient, and conductivity.

The energy content of an organism may also be altered—not by heating in the foregoing sense, but through an electrical mechanism which depends upon the presence in the organism of free charges or chemicals with dipole moments. Given either or both of these conditions, it is conceivable that the forced vibrations may lead to observable changes in the internal organization. If the homogeneous field intensity is sufficiently great, a small dipole may oscillate longitudinally without translation; i.e., it may alternately elongate and contract. The dipole movements are both longitudinal and translational in an inhomogeneous field. In translational vibration the dipole, as a whole,

moves small distances on either side of its mean position. We do not believe, however, that any of the frequencies employed were high enough to cause such free oscillations or "resonance" in our material.

Although a diversity of opinion prevails in respect to the question of nonthermal effects of high-frequency fields upon living material, the most assuring evidence appears to have come from those investigations which have dealt with small organisms, individuals, or cells whose normal environment can be simulated *in vitro* and in which the temperature of the surrounding medium is immediately assumed.

Kahler, Chalkeley, and Voegtlin (1929) exposed 150-400-cc. suspensions of *Paramecium caudatum* in electric and magnetic fields at 10 Mc and in electric fields at 75 Mc for periods of 1-4 hours. Also, Gale (1935) used the electric field at 15, 30, and 50 Mc for 1-6 hours on *P. caudatum* and *Chilomonas*. These workers found that the protozoa were destroyed at 41-43° C. when treated without temperature control. Slow direct heating with a water bath produced identical results. But when the suspensions were kept at sublethal temperatures by frequent chilling or by suspending the organisms in a non-conducting, nonheating medium (Kahler *et al.*), no after-effects were detected.

Jellinek (1936) attempted to exclude large-order heating effects by working with an extremely weak field. *Paramecium* and *Vorticella*, in single-drop cultures, were immobilized or destroyed immediately at 100 Mc. In view of his results with the same field upon other organisms, our own experiences with *Colpidium* in small quantities of fluid, and those of others on *Paramecium* at somewhat lower frequencies but greater field intensities, it seems probable that Jellinek's unusual results with the protozoa were due to other causes, perhaps contaminated glassware.

Kindred work on other microorganisms emphasizes the need for an efficient method of heat control for both organism and medium in order to exclude the possibility of selective or preferential heating.

Haase and Schliephake (1931) demonstrated that *Staphylococcus* cultures maintained at 55° C. in an electric field were destroyed in from 6 to 15 minutes, according to the frequency used. Thirty minutes were required for sterilization in a water bath at the same temperature. These authors believe that some peculiar effects upon the colloidal organization of the bacteria are involved.

By alternately exposing and cooling cultures of different bacteria, Whitney and Page (1935) observed that, when the media were not heated appreciably, the bacteria continued to live. Similarly, Breitweiser (1935) subjected bacteria on chilled media to intense fields at several frequencies. These produced luxuriant growths. After exposure to moderately strong fields on pre-warmed media, only a few scattered colonies of the bacteria were obtained. The authors attribute the lethal action to thermal changes in the media. Whitney and Page refer to the effect as a "thermal sterilization of the medium." They were also able to show that, at certain frequencies, temperature increased more rapidly within frog tadpoles than in the distilled water surrounding them, whereas a tap-water medium heated as readily as the animals. For colonies of bacteria or frog tadpoles it is therefore possible to obtain effects which are not proportional to the thermal changes in the medium. The experiments of Bachem (1935) on *Bacillus prodigiosus* and *Saccaromyces cerevisiae* are further suggestive. For a series of frequencies between 20 and 60 Mc, the rate of heating in these microorganisms was less than that of the fluids in which they were suspended. The reverse obtained between 60 and 85 Mc for the yeast and apparently above 85 Mc for the bacteria.

The question now arises: What light do our experiments throw upon the existence of an electrical mode of energy transfer distinct from heating? In order to avoid excessive temperature gradients in any part of the reacting system, our object was to eliminate, as far as possible, all temperature changes within the organism or medium during the course of treatment. To this end the material was prepared in the form of minute cylinders (capillaries) in which cooling is inversely proportional to, and heating a direct function of, the radius (Gale, 1935). The use of individual colpidia, instead of mass cultures, also excludes the so-called "antenna effect," which, as a function of population density, emphasizes the importance of numbers. Whitney and Page (1935), for example, demonstrated that organisms in groups were more responsive to high-frequency fields than single individuals.

Relatively large quantities of the rice-salt medium (pH 5.1) had a high rate of heating, as shown by a series of 40-minute determinations at each frequency setting. To cite one example: at 30 Mc the mean temperature change for 13.1 cc. of medium in the closed-off water jacket was 58.8° C., much greater than that required to bring the temperature of the medium (initially at 25° C.) up to the thermal death-point of *Colpidium* ($42 \pm 1^\circ$ C.). But at the same frequency, approximately 0.3 cu. mm. of the medium and one organism within an air-cooled capillary failed to heat appreciably, even after 6 hours of continuous treatment.

Observations on the colpidia exposed were fragmentary because direct examination of microorganisms within the field was not possible. The capillaries were examined within a few seconds after the power was turned off. In the water-cooled capillaries the behavior of the colpidia was identical with the controls in all respects. Their motor activities were normal, and their subsequent divisions were more or less synchronous with the controls. In the air-cooled capillaries slight differences were sometimes evident in the more rapid movements of the organisms and in their tendency to divide in advance of the controls. These were the only indications of temperature variations within the air-cooled capillaries.

SUMMARY

To investigate the possible existence of an effect of high-frequency fields on biological organisms, which cannot be shown to result entirely from heating, 129 colpidia were exposed to high-frequency electric and magnetic fields under the conditions of constant temperature and controlled environment. As a qualitative measure of change we used direct microscopic observations before and after exposure. For a quantitative measure we compared the mean rate of fission of the exposed organisms with the nonexposed controls otherwise treated identically. Three types of field were employed: a homogeneous electric field, a homogeneous magnetic field, and a highly inhomogeneous electric field.

From the results of these experiments we conclude that there is no large-order effect on the physiological organization of *C. campylum*, as evidenced by its subsequent division-rate, which can be attributed to a nonthermal mode of energy transfer from high-frequency electric or magnetic fields in the 13-60 Mc range of frequencies.

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NUTRITIONAL STUDIES OF PARAMECIUM MULTIMICRONUCLEATA.¹ I. QUANTITATIVE AND QUALITATIVE STANDARDIZATION OF THE FOOD ORGANISM

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CERTAIN experimental studies of allelocatalytic effects in protozoa feeding on bacteria point out a need for quantitative standardization of the food. McPherson, Smith, and Banta (1932) found that groups of *Paramecium caudatum* had a higher fission-rate than did singles when in a strong medium (high bacterial concentration) and that in a weak medium (low concentration of bacteria) the reverse was true. From this they conclude that conflicting results in the study of allelocatalysis may have arisen, in part at least, from differences in strength (food value) of culture medium used by different workers. Smith (1932), studying the strength of the culture medium as a factor in fission-rate of *P. caudatum*, also concludes that the concentration of the medium (presumably excess or deficiency of food) seems to be the predominating factor limiting the fission-rate. And Johnson (1933) in his studies on population densities in *Oxytricha fallax* has shown that many of the allelocatalytic results can be explained on a basis of the ratio existing between the infusorian and the bacterial population. It is therefore evident that some studies on the quantitative standardization of the foods of bacterial feeders are necessary in order that reproducible results may be obtained in future experimental work dealing with such organisms.

In most experimental studies on holozoic protozoa the "standard loop method" has been used for measuring quantities of food bacteria. This method, however, does not permit a rigid control of bacterial concentrations. Giese and Taylor (1935) endeavored to standardize the quantity of food for *Paramecium multimicronucleata* more accurately by comparing a mixture of the food organism, *Pseudomonas ovalis*, in their basic lettuce medium for culturing the ciliate, to a suspension of 0.025 per cent CaCO_3 . This served as a rough control. However, in view of the fact that certain protozoa among the bacterial feeders may now be physiologically standardized in large numbers because their excystment time is known, it is clear that a rigid control of the quantity of food is also desirable for further standardization of these experimental cultures. For that reason certain mechanical devices—the suspensiometer and turbidimeter—have been tested here for their accuracy in quantitative measurement of bacterial suspensions.

THE SUSPENSIMETER

Gates (1920) reported that the opacity of a bacterial suspension could be measured by the length of the column of the suspension required to cause the disappearance of a wire loop; and, by means of a simple formula, the measured opacity could be translated into terms of bacterial concentration per cubic centimeter and so made comparable to other suspensions of the same organism. An instrument for measuring the concentration of bacterial suspensions based on the principle of Gates is now on the market and is

¹ The writer is greatly indebted to Professor W. H. Johnson for suggesting this investigation and for his advice while carrying it out.

called a "suspensiometer." Such an instrument was tested and found inadequate for accurate quantitative measurement of suspensions of bacteria. Very milky suspensions of bacteria are required to obtain a sharp end-point for the disappearance of the image of the wire loop. The use of such dense bacterial suspensions makes it impossible to have consistent agreement between the numbers of bacteria present per cubic centimeter and the opacity of the suspension. In testing this device, all the bacterial suspensions were prepared from a single pedigreed strain of bacteria, which had been cultivated under uniform conditions for a definite period before use.

THE TURBIDIMETER

Richards and Jahn (1933) and Clifton, Mueller, and Rogers (1935) have described photoelectric turbidimeters which measured bacterial suspensions with a relatively high degree of accuracy. Fundamentally these devices are identical, as each employs a Weston Photronic photoelectric cell, the current output of which is essentially a linear function of illumination. Therefore, these cells were used to measure the light absorbed by a suspension of microorganisms.

In order to test the principle of the turbidimeter, an instrument was constructed which combined some features of each of the two described by the above-mentioned investigators. The apparatus consisted essentially of a light source, a 15.0×1.5 -cm. culture tube for the bacterial suspension, a Weston Photronic cell, and a microammeter.

The apparatus, patterned somewhat after the "vertical type" of Richards and Jahn, was housed in a Leitz-Wetzler microscope cabinet having inside dimensions of $5\frac{7}{8} \times 6 \times 11\frac{3}{4}$ inches. The light source (a 50-candle-power, 6-8-volt Mazda automobile lamp) was mounted in a Spencer substage lamp box containing a reflector. This metal lamp box in turn was secured to the inner side of the top of the cabinet so that the lamp filament was brought directly over the mouth of the culture tube inserted into a tight-fitting hole passing through a solid wooden block $3\frac{1}{4} \times 3\frac{1}{4} \times 4\frac{1}{2}$ inches. This block was supported on a shelf having a small 1.4-cm. beveled hole directly in line with that in the block. This arrangement permitted only light from the lamp passing through the entire length of the culture tube to fall on the Photronic cell, seated in a radio-tube socket, directly beneath and touching the shelf. The beveled hole in the shelf permitted the culture tube almost to touch the partially exposed face of the Photronic cell blocked off so that scattered light in dense suspensions would not fall over its surface. The culture tube was also held in position by a secondary shelf, which, sliding into place just beneath the lamp, helped to shut out any extraneous light which might otherwise pass below. Any other scattered light was absorbed by the black walls of the closed cabinet.

The intensity of the light source was maintained by means of a slide-wire resistance in series with the lamp and storage battery supplying the current for the circuit. The battery was continually charged by means of a General Electric Tungar battery charger during the course of the experiment.

The current generated by the Photronic cell was measured by a Weston Model 600 microammeter having a range of 0-500 microamperes. The position of the slide-wire resistance was adjusted so that the "standard" culture tube, when held in a definite position (noted by markings) and containing 20 cc. of sterile artificial pond water, gave a constant reading of 300 microamperes. When the intensity of the light was so regulated, the microammeter reading gave directly the percentage of light transmitted by any 20-cc. suspension substituted for the sterile medium in the "standard" culture tube.

The instrument was calibrated with suspensions of 4-day-old cultures of *Pseudomonas fluorescens* prepared by adding 8 "standard" loops to 20 cc. of sterile balanced-salt medium. The total number of bacteria in 1 cc. of each suspension was determined by the pour-plate method. Seven or 8 pour plates were always prepared for each count from the proper dilution of the suspension.

Clifton *et al.* (1935) reported for their turbidimeter that "a linear relationship between the bacterial counts and the logarithms of the microammeter reading was observed over a wide bacterial concentration range." Their findings were verified by the use of the instrument described above, to the extent that in a very narrow range of bacterial concentrations a straight-line relationship was found when the bacterial counts were plotted directly against the microammeter readings. Such linear relations were found to hold for the 4-day-old cultures of *Ps. fluorescens* grown at 26° C. on Difco Bacto nutrient agar.

As Richards and Jahn (1933) also reported that their turbidimeter estimates of yeast cultures were more accurate than the counts made with the haemocytometer, it is evident that turbidimeters do offer a means of standardizing more rigidly the density (quantity) of some food suspensions used in experimental protozoan studies. Therefore, their use should greatly enhance further standardization in experimental studies using protozoa having an established excystment time, as both the age of the protozoa and the quantity of a specific particulate food could be controlled.

As has already been stated, in most experimental studies on protozoa the standard loop method has been used to control the quantity of bacterial food required. In these studies it has been assumed that, even though there are slight variations in food quantity resulting from the use of the loop method, such variations do not influence the experimental culture, because some of these experimental animals seem to tolerate bacterial crowding within certain limits without effect upon their reproductive rate. Barker and Taylor (1931) in their investigations on *Colpoda* state:

If too many bacteria surround the protozoa, the latter are adversely affected as shown by a strongly reduced division rate. . . . In an exceedingly dilute suspension of bacteria the protozoa show reduction in size and division rate. . . . Between these two extremes is a relatively wide range of bacterial suspension densities in which the division rate and size of *Colpoda* are maximal and approximately constant.

Johnson (1933) in his studies on *Oxytricha* showed that there is an optimum concentration of food bacteria for the maximum rate of reproduction of the experimental animal. Using the wire-loop method, he found that suspensions of *Ps. fluorescens* in densities varying from 80 to 135 millions per cubic centimeter were optimal for a given number of this hypotrich under the conditions of his experiments. Johnson (1936) investigated the course of populations of *Paramecium* in different densities of bacteria and found that during the first 24 hours of culture there was no difference in the fission-rate of either singles or groups of five paramecia in either x or $5x$ concentrations of *Bacillus subtilis* in a controlled volume of the culture fluid. It is such findings as these which indicate that certain protozoa, among the bacterial feeders, can tolerate slight differences in food concentration in daily subculture without effect upon their rate of reproduction, and which justify the use of the wire-loop method for controlling the food quantities in experimental studies on these forms.

In order to determine more conclusively the advisability of the wire-loop method, it

was decided to ascertain if bacterial crowding over a long period has an effect upon the rate of reproduction in *Paramecium*. It was felt that if in x or $4x$ bacterial concentrations, representing densities of 2 and 8 standard 1-mm. loops, respectively, in 5 cc. of an inorganic culture medium, there were no differences in the average daily fission-rates of twenty paramecia in 1-cc. portions of these food suspensions, the standard loop method would be an adequate means of quantitatively standardizing the food supply of this organism in daily subculture. The slight variations in food quantity resulting in the use of this method would not equal the wide differences in the concentrations selected here.

MATERIALS AND METHODS

In order to establish reproducible conditions in an experimental study on the effect of different food concentrations on the fission-rate of *Paramecium*, it is necessary to use (1) a nonnutrient protozoan culture medium, (2) a pedigreed strain of bacteria as food qualitatively standardized, and (3) controlled optima for all physical environmental factors. The animals used in the present investigation were taken from a mass culture of *P. multimicronucleata* which Professor W. H. Johnson has had growing in his laboratory at Stanford University for many months at room temperature in an inorganic culture medium on *Ps. fluorescens*.

The non-nutrient culture medium used was comparable to that from which the animals were taken. This balanced physiological salt medium, first reported by Osterhout (1906), has been used successfully for the culture of various ciliates. It was employed by Barker and Taylor (1931) for the culture of *Colpoda cucullus* on *Ps. fluorescens*; by Johnson (1933) for cultivating *O. fallax* on *B. subtilis* and on *Ps. fluorescens*; by Gause (1934) for growing *P. caudatum* and *Paramecium aurelia* on *Bacillus pyocyaneus*; and later by Johnson (1936) for culturing *P. caudatum* on six strains of identified bacteria. The desirability of this non-nutrient medium is twofold: (1) it is reproducible; and (2) it will not support the growth of the bacterial inoculum, as shown by Johnson (1933). This non-nutrient salt solution was diluted 1:500 with triple-distilled water, so that its salt concentration was comparable to that of pond water, or 0.012 total salt. The formula is as follows:

H ₂ O	1,000	gm.	MgSO ₄	2.0	gm.
NaCl	52.0		KCl	1.15	
MgCl ₂	4.25		CaCl ₂	0.5	

One cubic centimeter of M/20 NaH₂PO₄ was added to each 30 cc. of the diluted salt solution for buffering, and the pH was brought to 6.8–7.0 with M/20 NaOH. The adjusted solution was then filtered through a Chamberland filter, as recommended by Johnson and Hardin (1938). After filtration, 5-cc. portions of the solution, in cotton-plugged tubes, were autoclaved at 15 pounds pressure for 20 minutes. These tubes were then ready for use.

For the present experiments the food organism was a pedigreed strain of *Ps. fluorescens* (U6) obtained from the bacteriology department of Stanford University. This food was qualitatively standardized throughout the experiments by cultivating the bacteria for a definite time before use at 26° C. on slants of the commercial Difco Bacto nutrient agar. This agar was selected in order to facilitate the establishment of reproducible conditions. To have the food organism a definite age (i.e., qualitatively standardized)

at the time of daily feeding of the protozoa, new subcultures of the bacteria had to be prepared daily and then incubated for the required period. The purity of these subcultures was regularly checked. At the time of feeding the paramecia, the aged bacteria were removed from the slants with a platinum loop 1 mm. in diameter and suspended in 5 cc. of the sterile salt solution. A level loopful was transferred each time, while the number of loops varied according to the experiment.

The ratio of the volume of the culture medium to the number of paramecia at the time of subculture was constant at 1 cc. to twenty animals, a ratio equivalent to one animal per drop from many pipettes. Sterile volumetric pipettes were used to carry 1 cc. of the bacterized culture medium into clean ground-glass Columbia culture dishes of 1 cc. capacity in which the ciliates were grown as isolation cultures.

The paramecia were subcultured daily by isolating twenty, with a sterile mouth pipette, from a similar culture started 24 hours earlier and grown in a moist chamber in an incubator maintained at 26°-27° C. by a thermostat. Counts of the remaining animals in each 1-day-old subculture were made under a dissecting microscope, with counting facilitated by the use of a mechanical hand counter. From the total number of progeny produced, the daily fission-rate was determined.

EXPERIMENTAL DATA

In these studies the food organism, *Ps. fluorescens*, was qualitatively standardized by being incubated either 4 or 30 days before use. And quantitative standardization, as stated above, required the use of an *x*, or 2-loop, concentration and a 4*x*, or 8-loop, concentration of each qualitatively standardized food. With the animal-medium ratio established at twenty animals per 1 cc. of each density of the conditioned food, several animal lines were prepared for study in a given experiment. The experiments were as follows:

- 5 lines of *Paramecium* in *x* density of 4-day-old food
- 3 lines of *Paramecium* in 4*x* density of 4-day-old food
- 3 lines of *Paramecium* in *x* density of 30-day-old food
- 3 lines of *Paramecium* in 4*x* density of 30-day-old food
- 5 lines of *Paramecium* in 2*x* density of 4-day-old food

Five lines of Paramecium in x density of 4-day-old food.—During the 4 weeks these five lines of *Paramecium* were carried in this 2-loop concentration of the 4-day-old food, their composite daily fission-rate averaged 1.2. Although there was some daily deviation from this average, the line cultures, for the most part, reproduced uniformly in relation to one another; and the protozoans in each were normal in appearance, in comparison with those in a mass stock culture. The average number of progeny per

TABLE 1

FIVE LINES OF 20 *Paramecium* IN 2-LOOP DENSITY OF 4-DAY-OLD FOOD

48	46	43	34	62	92	108	52	31	27	34	53	74	58	57	60
1.2	1.1	1.0	0.7	1.5	2.1	2.3	1.3	0.5	0.3	0.7	1.3	1.8	1.4	1.4	1.5
52	60	72	55	33	37	47	47	40	51	51	40	43	46	Av.	51
1.3	1.5	1.8	1.3	0.6	0.8	1.1	1.1	1.0	1.2	1.2	1.0	1.0	1.1	Av.	1.2

day of the five lines is given in Table 1, as is the average daily fission-rate. The viable bacterial concentration of a 2-loop, or *x*, density of 4-day-old food in 5 cc. of the culture medium averaged 74 million bacteria per cubic centimeter, as determined by the pour-

plate method, using ten test suspensions. The concentrations of the living organisms in these test suspensions were found to be 82, 76, 87, 81, 65, 80, 54, 74, 68, and 77 millions per cubic centimeter.

Three lines of Paramecium in 4x concentration of 4-day-old food.—After a period of 6 days, during which time one of these three lines had to be renewed, all the cultures began to flourish, as did those in the foregoing x concentration of the similarly aged food. And, except for the introductory period, these three lines maintained a relatively constant division-rate in 4x, or 8-loop, concentration. Over the 4 weeks that these lines were maintained, the composite daily reproduction-rate of the protozoans averaged 1.2 fissions. Since this rate is directly comparable to that of the foregoing five-line series in the x concentration of this same food, this would indicate that bacterial crowding within the limits of concentration of 2 to 8 standard 1-mm. loops has little or no effect upon *Paramecium* grown in isolation cultures as carried on in these experiments. In terms of viable bacteria, a 4x increase in concentration is equivalent to 296 million bacteria per cubic centimeter, judging from the average number of living organisms in an x concentration of this food. The history of the three line cultures of *Paramecium* in the 4x concentration of 4-day-old food is summarized in Table 2.

TABLE 2

THREE LINES OF 20 *Paramecium* IN 8-LOOP DENSITY OF 4-DAY-OLD FOOD

33	25	11	6	12	28	65	57	38	55	49	59	84	94	100	66
0.6	0.2	0.0	0.0	0.0	0.4	1.6	1.4	0.9	1.3	1.2	1.4	2.0	2.1	2.2	1.6
72	43	54	56	32	42	47	39	49	52	44	58	65	Av. 49		
1.8	1.0	1.3	1.4	0.6	1.0	1.1	0.9	1.2	1.3	1.1	1.4	1.6	Av. 1.2		

Three Paramecium lines in x density of 30-day-old food.—An x concentration of food of this age was found to contain, on an average, 12 million viable *Pseudomonas* per cubic centimeter of the culture medium. The three lines cultured in an x , or 2-loop, concentration of the food organism taken from 30-day-old slants were practically uniform in their daily division-rates. And there was little deviation from the average daily fission-rate of 1.0 divisions which the protozoans maintained during the 4 weeks they were subcultured. Table 3 summarizes these findings.

TABLE 3

THREE LINES OF 20 *Paramecium* IN 2-LOOP DENSITY OF 30-DAY-OLD FOOD

42	35	40	40	34	48	48	37	71	54	49	53	39	34	33	34
1.0	0.7	1.0	1.2	0.7	1.2	1.2	0.8	1.7	1.3	1.2	1.3	0.9	0.7	0.6	0.7
26	35	42	34	43	40	38	42	37	44	53	39	40	37	Av. 41	
0.3	0.7	1.0	0.7	1.0	1.0	0.9	1.0	0.8	1.1	1.3	0.9	1.0	0.8	Av. 1.0	

Three Paramecium lines in 4x concentration of 30-day-old food.—The paramecia in these three lines in standardized food suspensions of 30-day-old food in 4x concentration had an average division-rate similar to all the experimental lines so far considered. Judging from the experiment above, this concentration would contain 48 million living bacteria per cubic centimeter. During the 30-day period in which these protozoans were cultured in an 8-loop concentration of the food organism, the average daily fission-rate was 1.0. This average rate is identical with that of the three lines in the lesser concentration of the same food so qualitatively standardized. However, in the concentrated food suspension a greater deviation from the mean was noted. It was assumed

that this deviation was due to some other factor than concentration, since the average division-rate of the organisms in both the 2- and the 8-loop densities was comparable. The results are shown in Table 4.

TABLE 4

THREE LINES OF 20 *Paramecium* IN 8-LOOP DENSITY OF 30-DAY-OLD FOOD

68	60	63	62	45	64	45	51	42	34	35	32	33	36	36	33
1.7	1.5	1.5	1.5	1.1	1.6	1.1	1.2	1.0	0.7	0.7	0.6	0.6	0.8	0.8	0.6
38	40	51	36	34	32	25	25	24	25	31	35	37	33	Av.	40
0.9	1.0	1.2	0.8	0.7	0.6	0.2	0.2	0.2	0.2	0.5	0.7	0.8	0.6	Av.	1.0

Five Paramecium lines in 2x concentration of 4-day-old food.—In order to check the experiments so far considered, another experiment was carried on simultaneously in which the concentration of food was 4 loops and the food organism was 4 days old. Over a period of 3 weeks, in which five lines were subcultured, the composite average daily fission-rate of the paramecia was 1.1. As in the other experimental series herein described, the daily fission-rate of these particular lines was relatively uniform, with only slight deviation from the average mean, as seen in Table 5. The rate of 1.1 for the check experiment, being comparable to all the other lines carried in this investigation, permits the conclusion that bacterial crowding between 2 and 8 standard 1-mm. loops has no effect upon the division-rate of *P. multimicronucleata* when grown in isolation cultures under the reproducible conditions used in the present study. However, in other concentrations or conditions it may be that bacterial crowding has an effect upon *Paramecium*, as Johnson (1933) noted for another ciliate, *Oxytricha*, using *Ps. fluorescens* as food. The results of this control experiment are shown in Table 5.

TABLE 5

FIVE LINES OF 20 *Paramecium* IN 4-LOOP DENSITY OF 4-DAY-OLD FOOD

51	44	42	44	36	42	55	55	43	47	43
1.2	1.1	1.0	1.1	0.8	1.0	1.3	1.3	1.0	1.1	1.0
37	47	60	53	56	45	39	42	Av.	46	46
0.8	1.1	1.5	1.3	1.4	1.1	1.1	0.9	1.0	Av.	1.1

CONCLUSIONS

Agreement in experimental results in studies of protozoa which feed on bacteria is difficult to obtain if standardized conditions are not employed. The use of qualitatively and quantitatively regulated foods in such investigations, along with certain protistological techniques, should lead to reproducible results. Qualitative standardization, which implies that the food organism is at a given age at the time of being utilized, may be accomplished by certain bacteriological methods. The present investigation on quantitative standardization of the food supply of bacterial feeders shows that the quantity of food organisms may be regulated by the turbidimeter described. Its use, along with qualitatively standardized food, should facilitate further regulation of the experimental cultures in those investigations using protozoa capable of being physiologically standardized because their excystment time is known.

This investigation has also shown, for a specific case in which the experimental organism cannot be physiologically standardized in large numbers, that a rigid control of the quantity of food used is not necessary to obtain reproducible results, provided other

factors are controlled. *Paramecium multimicronucleata* grown under controlled environmental conditions on qualitatively standardized food was found to maintain a more or less uniform division-rate over an extended period even though the quantity of food varied considerably within a range which may be considered optimum under the conditions described. This was found to be the case when the food supply varied between 2 and 8 standard 1-mm. loops per 5 cc. of the inorganic culture medium and when the animal-culture medium ratio was twenty animals per 1 cc. at the time of daily subculture. These findings suggest that the wire-loop method is satisfactory for quantitatively measuring the bacterial foods of *P. multimicronucleata* when an optimum amount of food is employed, because the slight variation in food quantity resulting from its use would have no effect upon the division-rate of the organism. That this is true was shown by the fact that the concentration of viable bacteria may vary between 74 and 296 millions per cubic centimeter of 4-day-old food and between 12 and 48 millions in 30-day-old food without affecting the division-rate of *Paramecium* subcultured daily over a period of 4 weeks. It may also be concluded from the present findings that this protozoan can use *Ps. fluorescens* as food when the latter is between the ages of 4 and 30 days and is cultivated under the conditions described.

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SOME EFFECTS OF CORPUS LUTEUM EXTRACTS AND OF SYNTHETIC PROGESTERONE ON YOUNG MALE ALBINO RATS^{1,2}

(One plate)

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THE corpus luteum is an endocrine gland which, by means of its internal secretion, affects many processes in the postpuberal female physiology of mammals (Allen, Hisaw, and Gardner, 1939). These processes may be similarly affected in the absence of all luteal tissue by administration of crude potent luteal-tissue extracts or of pure crystalline progesterone, the so-called "hormone of the corpus luteum" (Allen and Corner, 1930; Corner and Allen, 1936). Whereas, all other sex hormones, both male and female, have been shown to have more or less bisexual properties, the corpus luteum hormone has long been considered specifically female in its action (Korenchevsky, 1937). With the advent of crude estrogen-free corpus luteum extracts (progestin, Allen and Meyer, 1933), pure crystalline hormone from extracts (Slotta, Ruschig, and Fels, 1934; Allen and Wintersteiner, 1934; Butenandt, Westphal, and Hohlweg, 1934), and synthetic progesterone (Fernholz, 1934; Butenandt and Schmidt, 1934; Butenandt and Westphal, 1934) it became more and more feasible to put to the test this assumption of sex specificity of the corpus luteum hormone. The experiments reported herewith were designed to determine whether definite effects follow the administration of progestin and progesterone in male rats.³

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² Presented before the American Society of Zoölogists at the Indianapolis meeting, December, 1937. Abstract in *Anatomical Record*, 70, Supp. 1, pp. 45-46.

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MATERIALS AND METHODS

We have used in these experiments: (1) sow corpus luteum extracts prepared in this laboratory ("Progestin I" and "Progestin II"); (2) sow corpus luteum extracts from the Upjohn Company ("Progestin III"); and (3) synthetic progesterone (Proluton, Schering). Progestin preparations had been subjected to treatment (Allen and Meyer, 1933) for removal of estrogens. Estrone (Theelin, Parke, Davis and Company) and testosterone (Oreton, Schering) were used for a comparison of their effects with those of corpus luteum hormone.

All corpus luteum hormone preparations were assayed on adult virgin female rabbits according to the techniques of Corner and Allen (1929) and Allen (1930).

Albino rats used in these experiments were of two kinds, namely, (1) an inbred strain derived from the regular colony, and (2) rats from the regular colony, used when inbred rats were not available. Female mice used as recipients of pituitary implants were from the small colony maintained in this laboratory for such purposes. Rabbits used for the assay of corpus luteum hormone were young mature virgin does purchased from a dealer and kept isolated in separate cages for a period of 1 month or more before treatment.

One hundred and ninety rats, 47 mice, and 21 rabbits were used in this investigation.

Methods standard in this laboratory for operation, injection, implantation of pituitaries, and autopsy were adopted. Histological methods were simple, involving paraffin imbedding and simple staining.

With the exception of assay work on rabbits, the principle of littermate controls has been used throughout. Analyses of organ weight data were carried out using Student's method.

EXPERIMENTAL RESULTS

PROGESTIN ON NORMAL YOUNG MALE RATS

To determine the possible effects of corpus luteum hormone on normal rats a wide dose range of progestin was tried on four litters of various ages (see Table 1). Young

TABLE 1

EXPT. No.	DOSE RANGE (Rb. U. [CORNER], DAILY)	PROGESTIN SAMPLE USED	INJECTION PERIOD (DAYS)		AGE AT AUTOPSY (DAYS)
			Age	Total	
I.....	1, 1/5, 1/20	I	38-51	14	52
II.....	1, 1/2	I	25-39	15	40
III.....	1, 1/2	I	17-31	15	32
IV.....	3	II	25-39	15	40

rats were used, for it is well known that young animals are more responsive to sex hormone treatments than are adults. In the first experiment a dose range of 0.05-1.0 rabbit unit (Rb. U.) (Corner) was tried, with the idea that some notion of threshold, and perhaps a quantitative difference in response, might be obtained should any effects of progestin be found. In the last experiment the largest dose which our limited supply would permit was given to one animal.

WEIGHT EFFECTS

Results of these experiments were, in the main, negative. Organ weights of treated animals differed from those of oil-injected littermate controls in but few cases. Even where some organ weight difference was observed, it could be considered only as suggestive because of the small number of animals used (one injectate at each dose-level). A brief outline of weight effects is detailed in Table 2.

TABLE 2

EFFECTS OF PROGESTIN ON ORGAN WEIGHTS ON THE BASIS OF ABSOLUTE WEIGHT AND PERCENTAGE OF BODY WEIGHT

EXPT. No.	PERCENTAGE OF DECREASE (-) OR INCREASE (+) IN ORGAN WEIGHTS OF TREATED VERSUS CONTROL ANIMALS											
	Body Weight	Testis	Pre- putial Glands	Ventral Pros- tate	Seminal Vesicles	Coagu- lating Glands	Peri- urethral Tissue	Cow- per's Glands	Adre- nal Glands	Thymus Gland	Thy- roid Gland	Pitui- tary Gland
I.	o*	o	—†	o	o	o	—	o	o	—	o	o
II.	o	o	—	o	o	o	—	o	o	o	o	o
III.	o	-42, -46	—	+51, +61	+60, +70	+59, +69	—	o	o	-19, -24	o	o
IV.	o	o	+50, +80	+99, +135	o	o	o	o	o	o	o	o

* Zero (o) indicates essentially no difference.

† Dash (—) indicates no weight taken.

A study of these data shows that in only one experiment (III), run from day 17 to day 32, was there any effect of injections on testis weight. This effect was a slowing of the growth-rate of the testis. At the same time there seemed to be a slowing of the growth-rate of the thymus and some stimulating effect on the growth of ventral prostate, seminal vesicles, and coagulating glands. In one other case (Expt. IV) the ventral prostate was distinctly heavier than that of the control. Preputial glands were heavier also. These weight differences were confined to organs which respond to androgens by similar changes. Therefore, they suggested a weak androgen-like effect of the injected material. None of the weight effects noted was very pronounced; and not enough animals were used to make the study of any response statistically significant.

EFFECTS ON SPERM MOTILITY

Sperm smears were taken at the time of autopsy of animals in Experiment I. In the animal which had received the highest dosage of progestin, spermatozoa were more numerous and more motile than in the animals receiving the lower dosages or in the oil-injected or untreated littermate controls. Animals in succeeding experiments were too young at autopsy to have spermatozoa in the epididymides or vasa deferentia.

EFFECTS ON GONADOTROPIC POTENCY OF PITUITARY

At the autopsy of animals in Experiments I and II the pituitaries were fixed for histological study. However, when the animals in Experiments III and IV came to autopsy, the pituitary gonadotropic potencies were tested by the implant method. In

Experiment III there appeared to be a definite depression in gonadotropic potency of pituitaries of both injected animals, as judged by ovarian and uterine weight differences in the implanted mice. In Experiment IV no such depression was apparent.

HISTOLOGICAL EFFECTS

Histological studies were made of certain organs from the experimental animals and their controls, to determine whether histological changes paralleled weight changes. A histological study of the testes was begun to determine what effect, if any, administration of progestin might have on sperm-head formation. Moore (1936) has shown that the time of appearance of sperm-heads in the testes of normal male rats of this colony is fixed at 33-35 days *post partum*. He found that this time of appearance was unaffected by injections of various gonadotropic preparations.

Experiments II, III, and IV were designed to test what effects administration of progestin might have on sperm-head appearance time. Two experiments (II and IV) were employed to test for any inhibitory action of the hormone on sperm-head formation within the dose range used. Injections were begun on day 25 and carried through day 39. Autopsy was on day 40. Oil-injected and normal littermate controls were run, and one normal control in each experiment was killed on day 33 to make sure that sperm-heads were not appearing extraordinarily early. It was decided that inhibition of sperm-head appearance would have to extend for about 5 days to be significant. Histological study showed that sperm-heads appeared by day 40 in all animals, including those treated with progestin. No sperm-heads were found in controls autopsied on day 33. Therefore, there was no apparent inhibitory effect from the dosages employed (0.5-3.0 Rb. U. daily) on the process of sperm-head formation.

Experiment III was planned to test for any accelerating action of progestin on sperm-head formation within the dose range employed. Injections ran from day 17 through day 31; autopsy was on day 32. Two dose-levels were used (0.5 and 1.0 Rb. U. daily). Oil-injected and normal littermate controls were run, and one normal control was allowed to live until day 36 to determine whether sperm-heads had formed by that time. Under these conditions there was no accelerating effect on sperm-head formation from administration of progestin. No sperm-heads were found in the testes of treated or control animals autopsied on day 32, whereas sperm-heads were found in the testes of the control autopsied on day 36. In the animal receiving 1 Rb. U. of progestin daily, not only was there no acceleration in the rate of sperm-head formation, but testis tubule diameters were definitely smaller than those of the normal littermates of the same age (ocular micrometer measurements). The testis weight was also far below normal (see Table 2).

The indications were, from these experiments, that progestin in the dosages employed had no effect on sperm-head appearance time but that testis weight and testis tubule diameter might be deleteriously affected.

Histological studies of other organs taken failed to reveal any striking differences correlated with progestin treatment. Organs studied included: (1) the sex accessories, such as ventral prostate, seminal vesicles, coagulating glands, periurethral tissue, Cowper's glands, and vas deferens; and (2) glands of internal secretion, such as adrenals, thymus, thyroids, and pituitary. Especial care was taken with the study of the pituitary, since Charipper (1934) had found what he called "pregnancy cells" in the pituitaries of normal and castrated male rats injected with 0.5 cc. of Lipolutin (Parke, Davis and

Company) for 15 days. According to Charipper, pregnancy cells are "large, ovoid, deeply eosin stained cells with homogeneous cytoplasm surrounding a usually eccentrically placed vesicular nucleus." Pituitaries of our experimental animals and their controls, and pituitaries of normal and pregnant female rats, were prepared according to Charipper's technique (Zenker fixation, Delafield's haematoxylin, and eosin). Eosinophilic cells were measured and studied for shape, for depth of stain, for homogeneity of cytoplasm, and for eccentricity of nucleus, without any special cell type having been found. Stein (1933, 1934) states that there are no special pregnancy cells in the rat. Other workers do not agree; nor do they agree on a definition of the term "pregnancy cell." Charipper (personal communication, 1937) now recognizes the error of the claims made in the 1934 paper.

EFFECTS OF CORPUS LUTEUM HORMONE ON CASTRATED YOUNG MALE RATS

It has been shown above that certain sex accessories of normal young males underwent a slight hypertrophy correlated with the administration of progestin. This and other findings suggested that the injected material might have a slight androgen-like effect. The normal male—even though immature—is useless as a test animal for androgens, because the animal's own gonads may be elaborating physiologically similar substances. Therefore, it was deemed advisable to cease all experiments on normals and to try injections of corpus luteum hormone into castrates to determine whether our preparations had any androgenic potency.

TREATMENTS

Littermates were castrated on the twenty-fifth day of life; and daily injections of corpus luteum hormone into some, of oil into others, were begun immediately. In all cases a normal littermate control was run. In most cases an uninjected castrated littermate control was run also. All treated animals and oil-injected controls were injected for 15 days, and all animals were autopsied on day 40. Progesterone was injected into four animals, in daily doses of 1, 2, 2, and 3 Rb. U. "Progestin II" was administered to one animal, 3 Rb. U. daily; and "Progestin III" was injected into one animal, also 3 Rb. U. daily. Six paired comparisons, therefore, were possible between treated castrates and their oil-injected castrated littermate controls.

WEIGHT EFFECTS

Organ weight data have been analyzed on the basis of such paired comparisons. Because body weight was affected, a new analysis was made to take this factor into account. That is to say, each organ weight was converted into percentage of body weight, and the paired comparisons were made anew. A summary of the two analyses is presented in Table 3, together with results of a determination by Student's method of the statistical significance of the results. Despite the fact that dose-level and character of preparation were not constant for all six injectates, the six paired comparisons were considered as a unit.

It will be noted that sex accessories were, in general, heavier than the corresponding organs of the castrated controls. This was particularly true of the ventral prostate, preputial glands, and periurethral tissue. Coagulating glands were consistently heavier in the treated animals, whereas seminal vesicle weights showed such consistency only when analyzed on the basis of percentage of body weight, and Cowper's glands showed it not at all. Adrenal glands showed some suggestion of being lighter in the treated

animals, and the thymus gland was consistently lighter. Thyroids were, on the average, slightly heavier in the treated animals, and pituitary weight followed body weight closely.

TABLE 3
STATISTICAL STUDY OF THE EFFECTS OF CORPUS LUTEUM HORMONE ON
THE ORGAN WEIGHTS OF CASTRATED YOUNG RATS

ORGAN WEIGHED	ON BASIS OF ABSOLUTE WEIGHT			ON BASIS OF PERCENTAGES OF BODY WEIGHT		
	Mean Δ^* (Per Cent)	Range of Δ (Per Cent)	P	Mean Δ (Per Cent)	Range of Δ (Per Cent)	P
Body weight.	- 0	- 17 to - 3	.013			
Preputial glands. . .	+ 82	+ 22 to + 124	.008	+ 99	+ 42 to + 131	.003
Ventral prostate. . .	+684	+300 to +1,329	.005	+746	+380 to +1,383	.003
Periurethral tissue. .	+ 73	+ 12 to + 183	.040	+ 90	+ 34 to + 228	.034
Coagulating glands	+ 43	+ 4 to + 82	.025	+ 57	+ 15 to + 109	.013
Seminal vesicles. . .	+ 19	+ 2 to + 64	.600	+ 31	+ 5 to + 90	.065
Cowper's glands. . .	+ 27	+ 9 to + 55	.053	+ 38	+ 6 to + 65	.023
Adrenal glands. . . .	- 18	- 26 to - 11	.001	- 9	- 24 to + 2	.065
Thymus gland. . . .	- 24	- 42 to - 5	.005	- 17	- 32 to - 2	.010
Thyroid glands. . . .	+ 11	+ 5 to + 38	.600	+ 23	+ 2 to + 66	.069
Pituitary gland. . . .	- 11	- 23 to - 2	.019	- 1	- 11 to + 12	.940

* Δ = Difference between injectate and control.

Since P represented the probability coefficient, and a P of .05 or less was taken as evidence of statistical significance, it may be concluded that there was a significant weight increase in the ventral prostate, preputial glands, periurethral tissue, and coagulating glands of treated castrates. The weight increase in Cowper's glands was probably significant, whereas the weight increases of the seminal vesicles approached significance only when analyzed on the basis of percentage of body weight. The thymus was significantly lighter in treated animals; the adrenals were probably significantly affected; and the body weight proved significantly lower in treated castrates. Thyroids and pituitary seemed to be less affected.

As an additional check on the validity of the results and to show that such results could not be the effect of random sampling, a series of twelve paired comparisons was made between castrated littermates receiving identical treatment (oil injections). The reference animal of each pair was chosen at the time of castration (day 25), in order to have a fixed basis of comparison and to avoid the possibility of later selection of animals in such a fashion as to make plus and minus weight differences balance. Autopsy was on day 40. Treatment of the data by statistical methods identical with those used above showed that there was a strikingly close agreement between littermates treated identically, and that in only one instance, and by only one method of analysis, did a probability coefficient fall below .05.⁴ Even this result fits well into the array of random significance figures which one would expect on the basis of chance.

⁴ Seminal vesicle, on the basis of absolute weight (P was .015). On the basis of percentage of body weight the P was .174. The range of the other probability coefficients was, on the basis of absolute weight, .260-.759, and on the basis of percentage of body weight, .201-.915.

Moore (1937) has called attention to the androgen measure available in littermate-controlled experiments, made possible by comparing the growth of end-organs in treated castrates with that of normal littermate controls. To determine what accessory sex organs were kept at or near the normal weight-level by corpus luteum hormone in the doses employed, the organ weight data were analyzed on the basis of paired comparison between treated castrates and their normal littermate controls. The following general conclusions were drawn: (1) preputial glands, ventral prostate, and periurethral tissue weights were maintained at or near the organ weight levels of the normal controls; and (2) coagulating glands, seminal vesicles, and Cowper's glands were definitely lighter than the corresponding organs of the normal controls.

COMPARISON OF WEIGHT EFFECTS OF CORPUS LUTEUM HORMONE AND OTHER SEX HORMONES

A comparison of organ weight responses was made between progesterone-treated castrates and testosterone-treated castrates. A graded series of testosterone was used in an endeavor to find a dose range giving effects comparable to those following progesterone treatment. Dilute solutions of testosterone in sesame oil were injected subcutaneously in daily doses of 0.03 to 0.10 mg. from castration on day 25 through day 39. Autopsy was on day 40. Comparison of the pooled data with data in Table 3 showed that testosterone, even in 0.10-mg. doses, did not cause a weight increase for preputial glands or ventral prostate equal to that produced by 1-3 Rb. U. of corpus luteum hormone. On the other hand, testosterone in 0.08-mg. doses caused about the same periurethral tissue increases as did corpus luteum hormone in the doses used; and as little as 0.03 mg. of testosterone daily caused more growth of coagulating glands, seminal vesicles, and Cowper's glands than did the doses of corpus luteum hormone used. Pituitary and thymus weights were slightly depressed by all doses of testosterone used, though the statistical significance of this finding was not tested. Adrenal and thyroid weights were not affected in either direction by such doses of testosterone at this stage.

The comparative effects of testosterone and progesterone treatment were analyzed in another way: comparison of the organ weights of testosterone-treated castrates with those of their normal littermate controls made possible deductions as to how much testosterone was necessary to keep the sex accessories at, or near, the normal weight-level. It was concluded that at this age, about 0.06 mg. of testosterone performed this function for most of the castrate sex accessories. A dose-level below 0.06 mg. failed to do this, and higher dose-levels gave heavier-than-normal accessory weights. All sex accessories except the ventral prostate responded to testosterone by comparable weight increases for any one dose-level. Ventral prostate weight was comparatively lower than that of any other accessory. Comparison of this action of testosterone with the action of corpus luteum hormone (see above) showed great differences in the stimulating potencies of the two hormones on different end-organs.

A better method of analyzing such differences in the two hormones, perhaps, is the method of prostate-*seminal vesicle* ratios used by Moore and Price (1937), Moore (1937), and Moore and Price (1938). The prostate-*seminal vesicle* ratio is obtained by dividing the total weight of the entire prostate complex (ventral prostate, coagulating glands, and periurethral tissue) by the weight of the seminal vesicles. It has been shown that the ratio decreases with age in normal rats, until it reaches 1/1 at sexual maturity, and that both androsterone and testosterone are able to produce any normal ratio in castrates, provided proper doses are used. Low doses produce high ratios, and vice versa.

Data for progesterone-treated and testosterone-treated animals and their controls were analyzed to determine the prostate-seminal vesicle ratio. Five castrates treated with 0.06 mg. of testosterone daily gave ratios averaging 6.5, or about the average for the normal controls (6.76). On the other hand, six castrates treated with corpus luteum hormone gave ratios averaging 18.3. It is to be remembered that each of the treated groups differed from the normals hardly at all in the weights of three accessories (preputial glands, ventral prostate, and periurethral tissue), but that the seminal vesicles of the progesterone-treated castrates were decidedly below normal in weight. This suggests that the corpus luteum hormone may adequately stimulate certain male end-organs without materially affecting others, in a fashion which is at variance with the action of testosterone.

A comparison was made of organ weight responses of progesterone-treated castrates and of estrone-treated castrates of similar age. A graded series of estrone-treated castrates was run, sometimes using littermates of the progesterone-treated animals, sometimes using different litters. Dilute estrone was injected in oil in graded doses (2-20 international units daily) from day 25 to day 39, with autopsy on day 40. In general, small increases, not correlated with dose-level, were found in organ weights of preputial gland, ventral prostate, coagulating gland, and periurethral tissue of treated castrates, as compared with similar weights of oil-injected castrated controls. Seminal vesicle weights showed increases correlated with dose-level; and weights of Cowper's glands, adrenals, thymus, thyroids, and pituitary showed no definite trend correlated with treatment.

In experiments with all three hormones, untreated castrated controls showed organ weights entirely comparable with those of oil-injected castrated controls. However, for the sake of uniformity, oil-injected castrated controls have been used for comparison with treated animals.

HISTOLOGICAL EFFECTS

Significant weight increases in certain accessory sex organs and weight decreases in the thymus of treated castrates led to the hypothesis that there might be an androgenic effect of corpus luteum hormone. Tissues taken at autopsy were divided into sex accessories and endocrine glands, and histological preparations were made. These two groups will be taken up in order.

SEX ACCESSORIES, OR ORGANS THAT MIGHT BE USED AS ANDROGEN INDICATORS

The ventral prostate.--The histology of the ventral prostate as an androgen indicator was worked out on the rat by Moore, Price, and Gallagher (1930); and a more careful investigation in young animals, both normal and castrated, was the work of Price (1936). She found that the normal prostate at 40 days is histologically similar to the adult gland, in that tubules are distended with secretion, gland cells are large and usually tall, and have "light areas" situated between the large basal round or oval nuclei and the free cell surfaces (see Fig. 1). These light areas are probably of significance in the physiology of secretion, for they are a constant feature of physiologically active cells, they disappear after castration, and they can be made to reappear with the administration of small doses of androgen. Consequently, they provide a sensitive test for the presence of androgens.

The prostates of animals castrated at 25 days and autopsied on day 40 showed striking differences from the normal picture (cf. Figs. 1 and 2). The tubular alveoli were smaller

and shorter. Tubular diameters were extremely reduced, as was also the amount of secretion within the tubule. Each gland cell was small, and practically cuboidal, with the nucleus near the center. Nuclei were smaller and more deeply stained. There was a relative (but probably not a true) increase in the intertubular connective tissue. Despite the fact that Price (1936) states that light areas persist in 30-40-day rats castrated during the first week of life, no light areas were found in animals castrated on day 25 and autopsied on day 40. Essentially the same thing was found by Greene, Burrill, and Ivy (1939).

In each comparison of normal control, castrated control, and corpus luteum hormone treated castrate, the ventral prostate of the treated castrate resembled, in all particulars, that of the normal control rat (cf. Figs. 1, 2, and 3). The tubules were distended with secretion; tubular diameters were fully as large as normal; and the gland cells were large, high columnar cells with basal nuclei. The nuclei were large and lightly stained. The proportion of tubular tissue and intertubular connective tissue was normal. Finally, light areas were found in the gland-cell cytoplasm between the nuclei and free cell borders.

Thus, it is apparent that the injected material, either corpus luteum extract or synthetic progesterone, acted as an androgen in building and maintaining the prostate in the normal secretory state.

Cowper's gland.—Heller (1930, 1932) worked out the histology of Cowper's gland as an androgen indicator. He says that by 40 days "the gland appears to have entered definitely upon its secretory activity." In adults, castration atrophy appears by 10 days, and is well advanced by 20 days, after castration.

In the normal 40-day-old rat Cowper's gland consisted of compound acini emptying into a central lumen. The entire gland was covered with a fibrous and muscular capsule. The central cavity and the gland duct were lined with a stratified epithelium. The acini were lined with tall columnar cells having small basally located, flattened nuclei, which were stained deeply with haematoxylin. The cytoplasm was very homogeneous and granular.

In rats castrated on day 25 and autopsied on day 40 Cowper's gland was much smaller than in the normal control. Considerable secretion still could be found in the central cavity, and there was a relatively greater amount of interacinous connective tissue and capsular material. Little secretory tissue was present. The few small acini were crowded between capsular connective tissue and the wall of the central cavity. Gland-cell height was much below that of the normal. Gland-cell nuclei were rounder and larger, less densely stained than in the normal. The condition was comparable to that of a much longer castration period in the adult (between 20 and 90 days).

In Cowper's glands of treated castrates the condition was intermediate between that described for the normal and for the castrated control. The secretory cells were higher, the acini were more numerous, and the interacinous connective tissue was relatively less prominent than in the oil-injected castrated control. Ocular-micrometer measurements showed that cell heights and acinar diameters were intermediate between those of the castrate and those of the normal control.

The vas deferens.—Vatna (1930) has worked out the histology and cytology of the vas deferens as an androgen indicator. Many changes due to castration were observed. He states that gross size is reduced, owing to a regression of the tunica muscularis. The amount of secretion within the lumen is diminished; and there is a reduction in epithelial cell height, a stratification of nuclei in the epithelial layer, obliteration of epithelial cell

walls, and a more or less complete loss of cilia-like structures from the epithelial surface. Where the "cilia" are not completely gone, they are short, matted, and disarranged.

In the castrate-control vas deferens at 40 days only some of the changes mentioned by Vatna were found. Obliteration of epithelial cell walls could not be seen in all, nor was there a difference in the amount of secretion within the lumen. Perhaps the more clearly defined changes from the normal in these animals were (1) reduction of the cilia-like processes, (2) reduction in gross size, and (3) apparent stratification of epithelial nuclei. Nuclei in the epithelial cells of castrates tended to be more basal than is normal.

Corpus luteum hormone treated castrates showed a histology and cytology of the vas deferens entirely comparable to the normal control. Lumina were large, and the epithelial cells retained their size and their long cilia-like processes. Gross cross-sectional size of the vas deferens was essentially that of the normal control. Nuclei of the epithelial cells were usually located about two-thirds of the distance from the free border of the cell to its base, as was the case in the normal controls.

Seminal vesicle.—The seminal vesicle cytology test has been suggested as a male hormone indicator by several workers. As worked out by Moore, Hughes, and Gallagher (1930), a positive test depends on the presence of secretion granules in sections of the seminal vesicle stained with Ehrlich's haematoxylin. Castration causes a disappearance of secretion granules from the adult seminal vesicle within 48 hours.

At 40 days the normal controls of the experimental series were at the beginning of their adult secretory state. Epithelial cells were not so high as in the adult, and secretion granules with halos were not so prominent or so frequent as in the adult. Secretion granules were, however, undoubtedly present by 40 days (Price, 1936, says they appear by day 36), and secretion could be seen in the lumen of the gland.

The condition of the castrate-control seminal vesicle at 40 days was distinctly different from that of the normal control. The cavity of the gland was much smaller and was not filled with a stainable secretion. The blind out-pouchings of the central cavity were not extensive. Secretory epithelium showed a severe castration atrophy. The cells were low, scarcely higher than the diameter of the nucleus; and no secretion granules were present.

Treated castrates showed essentially the same seminal vesicle histology and cytology as did the castrated controls. Epithelial cells were low, and there was practically no cytoplasm distal to the nucleus. In one case only was stainable secretion found in such a seminal vesicle. Search revealed a few secretion granules in a consistently higher epithelium. This was in RE175, an animal which received 3 Rb. U. of "Progestin III" daily, in dilute solution. Seminal vesicle weight increase was also the most pronounced (+90 per cent) of any animal in the series.

Middle lobe of the prostate (periurethral tissue).—Korenchevsky and Dennison (1935) have divided this prostatic tissue into three general subdivisions: lateral, dorsal, and central lobes. Moore (1939) mentions lateral and dorsal subdivisions only. Histological study was confined to the lateral subdivision. Acinar structure somewhat different from that of the ventral prostate was found. Cell heights of the normal and castrate gland did not differ appreciably, but acinar diameters decreased following castration. Similarly, effects of corpus luteum hormone were not on cell height, but rather on acinar diameters. Acinar diameters in the lateral subdivision of the prostate of treated animals averaged well above those of both the castrate and the normal control.

The coagulating gland.—Moore, Price, and Gallagher (1930) found that in the adult

the coagulating gland responds to sex hormone states and is an additional, though less satisfactory, androgen indicator. Response to androgens is merely one of epithelial cell size and acinar size.

A detailed comparison between normal and castrated controls of the experimental series, autopsied on day 40, failed to reveal any histological basis in the coagulating gland for distinguishing between the two categories of animals. Cell height measurements and acinar diameter measurements were not essentially different in the normal, castrated control or in castrated treated animals.

The preputial glands.—Preputial glands of adult rats respond to castration by certain well-defined histological changes, according to Korenchevsky and Dennison (1935). These are reductions in the number and size of the cells and alveoli. Cells become atrophic and more irregular. Vacuolization of the cytoplasm is coarser. Pyknotic nuclei are more numerous, and nuclei are packed closer together. Canals and ducts are smaller, are less ramified, and contain less secretion.

None of these changes were found in castrated controls of the experimental series, whether because of early age at castration and autopsy, because of short castration time, or because of some other factors we do not know. Neither was any histological change noted in the preputial glands of treated castrates.

ENDOCRINE GLANDS

Histological preparations of the adrenal gland, thymus, and thyroid were studied; but no striking changes attributable to either castration or corpus luteum hormone treatment were evident. Mammary gland and pituitary gland preparations have not been made.

A summary of the histological effects of corpus luteum hormone on castrates is presented in Table 4.

TABLE 4

Organ	Effects of Castration from Day 25 to Day 40	Effects of Hormone (1-3 Rb. U. Daily) into Castrates from Day 25 to Day 40
Ventral prostate.	Castration atrophy	Prevention of castration atrophy; light areas present
Vas deferens.	Castration atrophy	Prevention of castration atrophy
Cowper's glands.	Castration atrophy	Amelioration of castration atrophy
Seminal vesicles.	Castration atrophy	Slight repair in one case only
Middle prostate.	Decrease in tubular diameters	Tubular diameters larger than in castrate or normal controls
Coagulating glands.	No effects	No effects
Preputial glands.	No effects	No effects
Adrenal glands.	No effects	No effects
Thymus gland.	No effects	No effects
Thyroid glands.	No effects	No effects

EFFECTS OF IMPLANTS OF PITUITARIES INTO MICE

Three experimental series of animals had the pituitary gonadotropic potency assayed on 21-day female mice. No depression or augmentation of gonadotropic potency was observed which could be correlated with corpus luteum hormone treatment.

DISCUSSION

It has been seen in the preceding section that certain tests on male rats indicated a weak androgenic activity of corpus luteum extracts and of synthetic progesterone. A critical search of the literature published prior to the presentation of the preliminary report of this work failed to reveal claims of androgenic effects of corpus luteum hormone in males. In fact, most of the workers dealing with the subject of corpus luteum hormone effects in males have been unable to demonstrate any outstanding effects of hormone administration. Greene, Burrill, and Ivy (1939) have been able to confirm and to extend some of the conclusions presented in the preliminary report of this work.

All articles dealing with possible effects of corpus luteum hormone on male individuals may be classified into one of three general groups: (1) those claiming no effects, (2) those claiming effects which have been brought into question by later work, and (3) those claiming effects which have not been questioned. Of the first group, the work of Fels (1935), of Albrieux, Buño, Engel, and Morat6-Manaro (1936), of Fels (1936, 1937), and of Korenchevsky (1937) may be mentioned. As examples of the second group, the papers of Charipper (1934) and of Clauberg and Breipohl (1935) are cases in point. These authors claimed that corpus luteum extracts correct castration cells in the pituitaries of castrated rats. Fels (1935) and Hohlweg (1935), using larger doses of crystalline progesterone, failed to confirm this observation. They suggested that the results of the original workers may have been due to estrogenic impurities in their preparations. Charipper (1934) claimed also that "pregnancy cells" can be formed in the pituitaries of either normal or castrated male rats by administration of corpus luteum extracts. The work of Stein (1933, 1934) and the admission of Charipper (personal communication, 1937), already noted, dismiss this assertion. Examples of effects which have been reported but not questioned are the following: (1) Gardner and Hill (1936) have been able to produce mammary gland growth in normal and castrated male mice by corpus luteum treatment; (2) Zuckerman and Parkes (1936) found that the administration of progesterone to male monkeys causes no change in the prostate or other sex accessories but does tend to correct the pathology of the lining of the uterus masculinus which has resulted from injected estrone; (3) Burrows (1936) claimed that progesterone protects normal male mice, but not castrates, from the harmful effects of applied estrone; and (4) Nelson (1936, 1937) found that corpus luteum hormone is as effective as androgens in maintaining spermatogenesis in hypophysectomized rats but that no androgenic effects are evinced either by the injected material directly or by indirect stimulation to production of testicular internal secretion.

Whereas there were no previous claims in the literature that corpus luteum extracts or progesterone in males act as androgens, there were certain indications that such might be the case and certain findings which can be explained better, perhaps, by such an assumption. Steinach and Kun, as early as 1931, found that in the female guinea pig transformation of the clitoris into a penis-like organ results from any one of three kinds of treatment, namely, (1) X-irradiation of the ovaries, followed by luteinization; (2) administration of gonadotropic extract, followed by luteinization; or (3) administration of luteal extracts (into spayed or normal females). In all cases the level of corpus luteum hormone in the blood would be raised. Hill (1937*a*, 1937*b*, and 1937*c*) found that ovarian grafts in the ears of castrated male mice luteinize and that, where such grafts are present, the castrate accessories are repaired. Price (personal communication, 1937)

obtained "functional appearing" ventral prostate grafts when the hosts were either normal virgin female rats or female rats kept pregnant. Grafts are larger, on the average, in the latter hosts. Parkes (1937), using a very sensitive comb-growth test and extraction methods similar to our own for extracting corpus luteum hormone, found that certain ovarian extracts are slightly but definitely androgenic. Finally, as very good evidence, Steinach and Kun (1931) administered luteal extracts to young castrated male rats and produced normal-looking penis development. They thought that the corpus luteum produces not one hormone but two, namely, (1) a hormone for the production of a pro gravid uterine endometrium, and (2) a hormone identical with testis hormone. All these evidences fit into the general picture and are unified by the results of the present experiments.

A study of comparative effects of progesterone and testosterone suggests that the two may differ somewhat in their physiological expression, although both may act as androgens. A great deal more evidence is needed to corroborate this suggestion, but it does present some interesting implications. That androgens may differ among themselves in relative potency, and that a single androgen may show threshold differences with regard to different end-organs, is well known. It has also been strongly suggested (see Deanesly and Parkes, 1936; Korenchevsky, Dennison, and Brovsin, 1936; and Tschopp, 1936; and their citations) that androgens may differ among themselves as to their relative threshold differences—that is, a certain hormone may exhibit a "preferential" action on an end-organ out of all proportion to its effects on other end-organs, as compared with other androgens. Moore (Moore and Price, 1937; Moore, 1937; and Moore and Price, 1938) has brought this idea into question with his work on prostate-seminal vesicle ratios, and the problem is still unsettled. It is very certain that progesterone exhibits threshold differences for different end-organs in the castrated young male rat. Whether such differences are comparable with those exhibited by testosterone or are actually preferential for prostate tissue over and above those of testosterone, as they seem to be, remains for further work to decide.

There remains the question, still unanswered, as to whether progesterone acts as an androgen per se or is changed in the body by relatively simple chemical reorganization into an androgen. It is true that progesterone is more closely similar chemically to certain androgens, i.e., testosterone and androstenedione, than are these hormones to other androgens. The simplest change of progesterone to any of the known androgens would probably be to testosterone. However, since the physiological expression of the two (testosterone and progesterone) seems to be dissimilar, it is unlikely that this change takes place. It seems quite as possible that the progesterone acts per se as a weak androgen.

It is not unlikely that still other weight responses and histological changes may be found to result from progesterone treatment when more extensive work is done, using more animals and larger doses of the hormone. When considered on the basis of percentage of body weight, the adrenals and thyroids of treated castrates showed weight decreases approaching a statistically significant value. Also, it is possible that with larger doses the seminal vesicles may be affected in the same manner as are the other sex accessories.

That sex accessories, which would be expected to act in similar fashion under sex hormone stimulation, showed, in general, weight increases, whereas other organs showed no change in weight and still others showed weight decreases, adds to the dependence to

be put in the results. Confirmation is to be had in histological changes correlated with treatment. All positive effects indicate a weak androgen-like activity of progesterone.

That the effects seen are due to progesterone alone, and not to any other substance, seems indicated by the following facts: (1) controls injected with plain oil showed none of the effects; (2) all extracts used were treated to remove estrogens; (3) most of the work on castrates was done using synthetic crystalline progesterone, both the extracts and pure progesterone giving the same results; and (4) the comparison of progesterone effects with effects of estrone showed a decided qualitative difference in the two hormones.

Korenchevsky (1937) has advanced the idea that sex hormones can be classified into one of three general categories, namely, (1) monosexual, (2) partly bisexual, and (3) true bisexual hormones. Most hormones fall into the bisexual groups. In fact, progesterone alone was placed in the monosexual category as a purely female hormone. This idea has been controverted by the present experiments, and it may now be said that we know of no purely monosexual hormone.

SUMMARY

1. Results of administration of corpus luteum extracts to normal young male rats suggested a weak androgenic action of the preparations.

2. Within the dose ranges employed ($\frac{1}{2}$ –3 Rb. U. [Corner] daily for 15 days) there was no slowing or acceleration of the rate of sperm-head formation due to the injected corpus luteum extracts.

3. Progesterone or corpus luteum extracts injected (1–3 Rb. U. [Corner] daily for 15 days) into castrated young male rats caused (1) a significant weight increase for preputial glands, ventral prostate, periurethral tissue, coagulating glands, and possibly Cowper's glands; (2) a significant weight decrease for body weight and thymus gland; and (3) no decided weight change for seminal vesicles, adrenals, thyroids, and pituitary.

4. Difference in grade of weight response of the various sex accessories to progesterone may be attributed to a threshold phenomenon.

5. Comparison of weight effects of progesterone and testosterone suggests a difference between the two with respect to relative thresholds for prostatic tissue and the seminal vesicle.

6. Castrated sex accessories which responded histologically to progesterone and to corpus luteum extracts by amelioration of castration atrophy were, in order of decreasing amelioration: ventral prostate, vas deferens, lateral lobe of middle prostate, Cowper's glands, and seminal vesicles. Accessories which responded histologically neither to castration nor to injections were the coagulating glands and preputial glands. Adrenals, thymus, and thyroids showed no histological changes to castration or to corpus luteum hormone injections under the experimental conditions.

7. No difference in pituitary gonadotropic potency was observed between injected castrates and their controls.

8. All positive effects of progesterone and corpus luteum extracts in young male rats, castrated or normal, pointed to an androgenic potency of these preparations.

9. Since results of progesterone and corpus luteum extracts on castrates were entirely similar, and since injections of estrone gave very dissimilar results it may safely be concluded that all results were due to the administration of the corpus luteum hormone and not to any other hormone or impurity.

10. Prior to these experiments progesterone was the only sex hormone for which bisexual effects had not been demonstrated. Now it has been shown to have effects on accessory sex organs in the male.

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PLATE I



FIG. 1

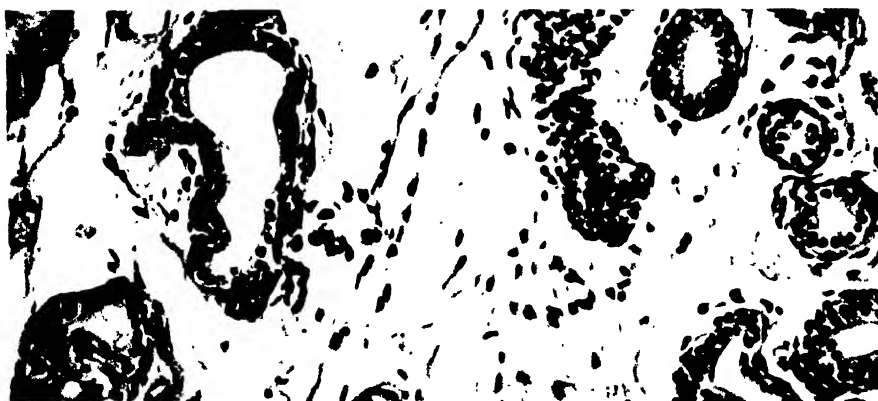


FIG. 2



FIG. 3

PLATE I

FIG. 1.—Section of a normal ventral prostate at 40 days. $\times 260$.

FIG. 2.—Section of the ventral prostate of a littermate, castrated from day 25 to day 40. $\times 260$.

FIG. 3.—Section of the ventral prostate of a littermate castrated, and injected with 2 mg. of progesterone daily, from day 25 to day 40. $\times 260$.

EFFECTS OF LOW ENVIRONMENTAL TEMPERATURE ON THE THYROID AND ADRENAL GLANDS OF THE GROUND SQUIRREL, *CITELLUS TRIDECIMLINEATUS*¹

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IN 1931 a group of workers at the zoölogical laboratories of the University of Chicago, including the writers of the present paper, began a systematic investigation of the reproductive cycle of the thirteen-lined ground squirrel, *Citellus tridecemlineatus*, as well as of the factors underlying and influencing this reproductive cycle. The results of this investigation were presented, in condensed form, in a general report (Moore *et al.*, 1934) and were subsequently elaborated by Wells (1935a) for the male and by Simmons (1934) for the female. The sexual cycle of *C. tridecemlineatus* was described, and evidence was presented that among the endocrine factors involved in the manifestation of reproductive periodicity in this wild, annual-breeding mammal the hypophysis played a significant role. In an attempt to analyze the environmental conditions which in turn influence the hypophysis, the effect of low environmental temperatures upon the animal, among other factors, was investigated. Peculiarly enough, it was found that animals of either sex placed during the season of breeding capacity (April-May) in a cold room with a constant temperature of $+4^{\circ}$ C. tended to remain on a high level of sexual development so long as they were exposed to the colder environment, whereas normally they undergo sexual retrogression during the summer. This effect of low environmental temperature appeared striking and stimulating to further investigation. Thus, the possibility of observing corresponding effects of low environmental temperatures upon the thyroid and adrenal glands suggested itself as an object of especial interest in the study of endocrine interrelationships, particularly in view of the fact that data on the seasonal cycle in the thyroid and adrenal glands of *C. tridecemlineatus* have previously been obtained by the senior author (1934 and 1935, respectively) and were thus available for comparison.

MATERIALS AND METHODS

This investigation was conducted on 39 males (24 experimental and 15 controls) and 26 females (13 experimental and 13 controls). The experimental animals were captured in the field during April and early in May, i.e., during the period of high sexual development and breeding capacity, and placed immediately thereafter in an electrically refrigerated room in which the temperature was maintained thermostatically at $+4^{\circ}$ C. with a high degree of constancy. The control animals were also collected in the field during the same season and kept in the laboratory at room temperature. The animals

¹ The writers wish to acknowledge their indebtedness to Dr. Carl R. Moore for his helpful guidance and critical reading of the manuscript; to Dr. W. C. Allee for placing at the writers' disposal the cold-room facilities of the Whitman Zoölogical Laboratory; and to Miss Ruth Berman for her valuable technical assistance.

of the cold room were isolated from each other in small individual cages, so that they were unable either to cuddle together and thus reduce the loss of their body heat or to devour their hibernating cagemates. The controls were caged in groups of 4-6. The food of both the experimental animals and the controls consisted of mixed grain with added "rabbit chow checkers." These "checkers" are prepared by the Purina Company and contain 16.45 per cent of protein. Ground squirrels seem to thrive on this diet and show evidence of good health and satisfactory physical condition. The relative humidity of the cold room² was 62.7, whereas in the laboratory it registered only 38.2. It is doubtful, however, whether the difference in humidity influenced appreciably the results of the experiment, since animals previously kept under various conditions of humidity had not shown any observable physiological or developmental differences.

To exclude possible effects of darkness, 14 males and 8 females of the experimental lot were subjected to ultra-violet radiation for 4 hours daily for periods ranging from 28 days to $7\frac{3}{4}$ months in the otherwise dark cold room. Two Westinghouse mercury-vapor lamps, type g-5, with an output of 15.23 microwatts per square centimeter,³ were used. All experimental animals were sacrificed on the day of their removal from the cold room, along with several controls.

At autopsy the reproductive organs, the thyroids and the adrenals were removed, weighed fresh, and prepared for histological study. Usually one thyroid and one adrenal from each animal were fixed in Bouin's fluid, while for the remaining thyroid and adrenal Bensley's formalin-Zenker was used as fixative.

Omission of 95 per cent and absolute alcohols from the dehydration process of the thyroid and adrenal and the substitution of aniline oil for these alcohols ($\frac{2}{3}$ of 70 per cent alcohol + $\frac{1}{3}$ of aniline oil for 1-2 hours, $\frac{2}{3}$ of aniline oil + $\frac{1}{3}$ of 95 per cent alcohol for 1-2 hours, and then pure aniline for 1-2 hours) were found helpful for satisfactory sectioning. The tissues were imbedded in a mixture of 60° paraffin, 1 per cent rubber, and 5 per cent Bayberry wax, and sectioned at 4-5 μ . The staining was done chiefly in Krichesky's modification of the triple Mallory mixture.

For the determination of the relative width of the zones in the adrenal cortex, an ocular micrometer was used.

RESULTS

The prolonged exposure of the thirteen-lined ground squirrel to a practically constant temperature of +4° C. caused the animal to exhibit hibernation tendencies. However, in no case was hibernation sustained and uninterrupted. Various degrees of intermittency in hibernation were observed. Some animals were usually in torpor when periodic observations were made, while others were never seen in the hibernating state.

Loss of body weight was, as is to be expected, directly proportional to the relative length of time spent by a given animal in hibernation. The mean body weight of 24 experimental adult males was 178 gm., while in 15 comparable control adult males it amounted to 225 gm. (Table 1). The average body weight of the 13 experimental females (all adult) was 169 gm., whereas in the comparable controls of this sex the corresponding average was 198 gm. (Table 2).

The effects on the reproductive system of prolonged exposure of males of the thirteen-

² A Tyco's wet-and-dry-bulb autohigrometer was used.

³ At a point on the axis of the aluminium reflector 1 meter from the source.

TABLE 1

COMPARISON OF BODY WEIGHT, SEXUAL CONDITION, AND WEIGHT OF
THE THYROID AND ADRENAL GLANDS IN COLD-ROOM
AND CONTROL MALES

A. EXPERIMENTAL MALES

Number of Animal	Placed in Cold Room	Sacrificed	Body Weight (Gm.)	Weight of Testis (Gm.)	Spermatozoa in Epididymis*	Weight of Thyroids (Mg.)	Weight of Adrenals (Mg.)
1024....	4-13-35	8-27-35	160	0.7250	++++
1025....	5-27-35	8-27-35	184	0.5074	++++
1085....	4-13-35	11- 6-35	135	0.6872	++++
1086....	4-13-35	11- 6-35	160	0.6000	++++
1087....	4-13-35	11- 6-35	165	0.5212	++++
1088....	4-13-35	11- 6-35	139	0.5108	++++
1170....	4-13-35	4-22-36	170	0.6470	++++	22.4	21.0
1171....	4-13-35	4-22-36	180	0.4950	++++	43.0	22.2
1268....	4-18-36	8-30-36	211	1.0190	++++	22.6	31.2
1269....	4-18-36	8-30-36	195	0.8700	++++	22.0	33.8
1272....	4-18-36	9-29-36	180	0.8360	++++	17.0	40.0
1273....	4-18-36	9-29-36	160	0.7650	++++	26.2	22.0
1289....	4-18-36	10-31-36	211	0.7220	++++	50.0	43.0
1290....	4-18-36	10-31-36	190	0.5870	++++	26.0	16.0
1292....	4-18-36	12- 2-36	175	0.7400	++++	39.6	18.8
1294....	4-18-36	12-24-36	200	0.6745	++++	29.0	21.0
1295....	4-18-36	12-24-36	180	0.3170	o	20.0	32.0
1322....	4-18-36	1-29-37	190	0.5874	++++	39.7	26.5
1362....	4-18-36	2-25-37	178	0.6144	++++	25.6	21.1
1377....	4-18-36	4-23-37	187	0.6578	++++	19.4	31.6
1378....	4-18-36	4-23-37	187	0.6273	++++	20.3	19.0
1379....	4-18-36	4-23-37	190	0.6195	++++	17.4	14.3
1400....	5-11-37	11-10-37	170	0.5138	++++	53.8	21.6
1401....	5-11-37	11-10-37	155	0.4351	++++	17.8	21.0
Mean.....	178	0.6370	27.3	25.9

B. CONTROL MALES

Number of Animal	Sacrificed	Body Weight (Gm.)	Weight of Testis (Gm.)	Sperma- tozoa in Epididy- mis*	Weight of Thyroids (Mg.)	Weight of Adrenals (Mg.)
1211....	7-18-36	295	0.1412	o
1212....	7-18-36	235	0.1192	o
1215....	8-30-36	210	0.1800	o	35.9	19.4
1217....	8-30-36	275	0.1182	o	24.5	23.7
1218....	8-30-36	205	0.1574	o	26.1	27.8
1220....	8-30-36	195	0.1414	o	15.3	24.4
1221....	8-30-36	235	0.2558	o	17.0	29.5
1270....	8-30-36	342	0.2302	o	12.4	21.8
1271....	8-30-36	190	0.1268	o
1274....	10-14-36	205	0.2450	o
1275....	10-14-36	120	0.1200	o
1288....	10-31-36	215	0.3160	o	24.4	54.2
1291....	12- 2-36	258	0.2940	o	21.8	27.8
1293....	12-24-36	205	0.5850	o	22.0	21.0
1321....	1-29-37	190	1.1720	++	21.5	26.8
Mean.....	225	0.2801	22.1	27.6

* Estimate of quantity and motility of spermatozoa in the epididymis ranges from o (spermatozoa lacking) to ++++ (spermatozoa abundant, vigorously motile).

lined ground squirrel to low environmental temperature have been reported and described by the writers elsewhere (Wells, 1935*b*; Wells and Zalesky, 1940). For the pur-

TABLE 2
COMPARISON OF BODY WEIGHT, SEXUAL CONDITION, AND WEIGHT OF
THE THYROID AND ADRENAL GLANDS IN COLD-ROOM
AND CONTROL FEMALES

A. EXPERIMENTAL FEMALES

Number of Animal	Placed in Cold Room	Sacrificed	Body Weight (Gm.)	Weight of Ovaries (Mg.)	Weight of Uterus (Mg.)	Condition of Vaginal Membrane	Weight of Thyroids (Mg.)	Weight of Adrenals (Mg.)
B-3.....	5-3-34	11-23-35	176	31.5	2,320.0	open	37.4	39.8
B-64.....	5-6-36	12-16-36	168	14.4	828.0	open	30.8	28.6
B-65.....	5-6-36	12-16-36	187	38.0	402.0	open	35.7	20.0
B-66.....	5-6-36	12-16-36	187	28.8	833.0	open	32.0	14.0
B-68.....	5-6-36	12-17-36	165	35.0	455.0	open	22.8	41.0
B-69.....	5-6-36	12-17-36	193	15.0	330.0	open	39.8	12.0
B-70.....	5-6-36	12-17-36	205	33.8	525.2	open	36.6	37.5
B-71.....	5-6-36	12-17-36	180	27.4	604.6	open	34.0	20.0
B-72.....	5-6-36	12-17-36	202	31.0	667.0	open	30.4	14.6
B-94.....	5-3-37	12-1-37	115	12.2	541.8	open	35.8	21.8
B-95.....	5-3-37	12-1-37	140	16.8	584.6	open	40.2	21.0
B-97.....	5-3-37	12-4-37	117	15.6	335.8	open	37.0	26.0
B-98.....	5-3-37	12-4-37	140	25.0	781.0	open	30.3	27.0
Mean.....			169	25.0	708.4		34.8	24.9

B. CONTROL FEMALES

Number of Animal	Sacrificed	Body Weight (Gm.)	Weight of Ovaries (Mg.)	Weight of Uterus (Mg.)	Condition of Vaginal Membrane	Weight of Thyroids (Mg.)	Weight of Adrenals (Mg.)
B-63.....	12-16-36	180	16.0	242.0	closed	24.9	12.4
B-67.....	12-16-36	212	20.0	118.0	closed	21.0	19.0
B-73.....	12-17-36	205	20.2	155.8	closed	28.3	16.8
B-74.....	12-17-36	188	13.2	90.8	closed	23.8	11.0
B-75.....	12-17-36	190	15.0	61.0	closed	24.6	11.8
B-76.....	12-18-36	167	16.6	409.4	closed	36.0	20.0
B-77.....	12-18-36	205	17.2	226.8	closed	23.8	11.4
B-78.....	12-18-36	216	21.4	128.6	closed	24.2	16.2
B-93.....	12-1-37	164	13.6	120.8	closed	27.0	17.6
B-96.....	12-4-37	200	13.6	114.6	closed	29.6	20.8
895.....	12-2-34	252	17.7	144.9	closed	11.8	20.0
892.....	11-16-34	148	14.2	116.2	closed	10.4	17.6
893.....	11-16-34	129	13.4	110.0	closed	21.2	11.0
Mean.....		198	16.3	156.8		23.6	15.8

pose of the present investigation it will suffice to note that the males subjected to low environmental temperatures did not undergo sexual regression, which normally takes

place in the male during the early summer and which persists until new seasonal development is initiated in December or January.

Also in the female, as previously reported (Moore *et al.*, 1934; Simmons, 1934), corresponding effects were observed. Females placed in the cold room early in May, i.e., at or near oestrus, exhibited at autopsy in December a state of high sexual development, whereas in nature or in the laboratory they are sexually regressed from August to February.

The mean weight of the thyroid (Table 1)⁴ in the experimental group of adult males (27.3 gm.) shows an increase of 24 per cent over the corresponding average in the control lot (22.1 mg.). This increase, however, is statistically not very significant, and the number of males whose thyroid weight was available was small (18 experimental and 10 controls). The establishment of the possible significance of the increase in weight of the thyroid gland under conditions of low environmental temperatures would require a larger series, in view of the normally wide range of variation in the weight of the thyroid gland of the ground squirrel.

Histologically, nothing could be observed to justify a significant increase of weight in the thyroid gland of the male ground squirrel under the influence of low environmental temperature. The thyroid of the experimental animals resembled in every detail the thyroid of the controls, except perhaps for the tendency toward a larger number of small follicles displayed by some experimental animals.

In so far as could be determined from histological study, the glands of the experimental males were essentially in the same phases of activity as those of the laboratory controls. They were of two types, either one or the other predominating within the limits of a given gland. Most common was the "resting" gland, with its low epithelium, compressed, elliptical nuclei, distended follicles, and dense colloid. Occasionally the "storing" gland was encountered. In this type of gland the epithelium is largely low cuboidal or cuboidal, the nuclei of the epithelial cells are spheroid, the apical portion of the epithelial cells contains colloid floccules, and the colloid is usually less dense than in the "resting" gland. The "storing" gland is no doubt secreting through the apical pole of the cell into the lumen of the follicle, thus storing colloid for the future need of the organism. Vacuoles of resorption are exceedingly rare in either the "resting" or the "storing" thyroid. Whenever found, they are located in one or several of the smaller central follicles which, as a rule, are more active than the peripheral acini.

The females exposed to cold had significantly heavier thyroids than laboratory controls of the same sex. The mean weight of the thyroid gland of the 13 experimental females was 34.8 mg., while the corresponding average for the 13 controls was 23.6 mg., the increase in weight in this case amounting to 47 per cent (Table 2). Again, however, this increase in weight could not be ascribed to heightened secretory or excretory activity of the glands, in so far as it could be judged from histological appearance. The same "resting" and "storing" glands as were observed in the thyroid of the male were likewise encountered here. However, the tendency shown by the thyroid of some experimental males toward an increase in number of small follicles was even more pronounced in the cold-room females and may well account for their heavier thyroids.

The histological structure of the thyroid of the female did not differ from that of the male. The "resting" follicle displayed the same squamous epithelium, compressed nuclei, and abundance of stored dense colloid. The "storing" follicle was characterized

⁴ In every case both thyroids were weighed fresh.

by a similar cuboidal epithelium, by colloid floccules or globules in the apical portion of the epithelial cell, and by a usually thinner colloid. In two experimental females the thyroid colloid was quite thin; it stained a faint blue with Mallory and contained fine droplets of still lighter material. Nevertheless, this type of colloid was found also in the thyroid of one control female, so that it cannot be considered a result of the treatment.

It would seem, therefore, that neither the lower environmental temperature nor the prolongation of the high state of sexual development resulting from extended exposure to lower environmental temperature had any visible effect upon the thyroid gland of the male or female *C. tridecemlineatus*. Nor did exposure of the animal to ultra-violet radiation appear to influence visibly the thyroid gland of either sex.

Prolonged and continuous exposure of the male ground squirrel to a temperature of $+4^{\circ}$ C. failed to affect the weight of the adrenal gland. The average weight of the adrenal in the experimental series is 25.9 mg. and in the control lot 27.6 mg. (Table 1). Histologically, however, definite effects of the treatment were demonstrable.

In a study of the seasonal changes taking place in the adrenal gland of the same species, conducted by the senior writer (1934), it was shown that in the male the outer reticular subzone of the adrenal cortex (noncommittally termed by the writer "reticularis A") becomes particularly wide, well developed, and conspicuous during the peak of reproductive activity, i.e., during April and May. The cells of this subzone are characterized by large size, high cytoplasm-nucleus ratio, and by a deeply staining area surrounding the nucleus and usually leaving a narrow, light perinuclear ring and a similar peripheral ring. The reticularis A cell is apparently a highly specialized cell which has gradually developed from a cell of the fasciculate zone. Its physiological significance has not been determined.

Concomitantly with this widening and accentuation of cellular characteristics of the reticularis A during the period of high sexual activity, there is a general expansion of the reticular zone, partly at the expense of the fasciculata, as well as an expansion of the cortex in general. The cortex-medulla ratio as calculated by the planimetric method (Zalesky, 1934) is highest during the period of breeding capacity. The weight of the gland is also highest during this phase of the annual reproductive cycle.

Similar tendencies are displayed by the male ground squirrel subjected to the cold-room treatment. The reticularis A, on the whole, shows greater development, both qualitatively and quantitatively, in the adrenals of the experimental animals. The adrenals of some controls sacrificed during November and December lack a reticularis A altogether. This expansion of the outer reticularis in the adrenals of the experimental animals occurs at the expense of the fasciculate layer. The weight of the gland as a whole does not increase. Nor is the cortex-medulla ratio consistently altered. In one adult experimental male it amounted to 5.9:1 and in another to 9.8:1, as compared to 5.7:1 in the standard adult control.

The average width of the reticularis A in the control animals was slightly over 3 cells, whereas in the experimental animals it was over 8 cells.

In the female these changes were even more pronounced than in the male. This is equally true of the normal seasonal cycle in the adrenal gland of the female. Whereas in late autumn and winter, i.e., during the period of anoestrus, there is usually no trace of reticularis A in the adrenal cortex, in April and May, i.e., during oestrus and pregnancy, this subzone may reach 12-15 cells in width and appear as the most con-

spicuous layer in the adrenal cortex. Similarly, the range of seasonal variations in the weight of the gland is considerably wider in the female than in the male. Thus, during the period of sexual inactivity the mean weight of both adrenals of the female is 12.2 mg., while at oestrus it amounts to 22.3 mg., and during pregnancy to 31.0 mg.

The cortex-medulla ratio also shows, in the spring, an increase to 10.9:1 from the November low of 5.2:1.

Similar differences were observed in the present investigation between the control females and the females whose high degree of sexual development has been prolonged by exposure to an environmental temperature of $+4^{\circ}\text{C}$. The mean weight of the adrenal gland of the 13 control females was 15.8 mg.; the corresponding average for the 13 experimental females amounted to 24.9 mg., representing an increase of 58 per cent (Table 2). The cortex-medulla ratio in a typical experimental female was found to be 9.6:1; whereas the adrenals of all controls showed a virtual absence of the reticularis A subzone, in the adrenals of the experimental animals this subzone was 4–8 cells wide. The cells of the fasciculate zone were likewise larger and more highly developed in the adrenals of the experimental females than in those of the controls of the same sex.

In either male or female the zona glomerulosa of the adrenal cortex, as well as the adrenal medulla, did not seem to be visibly affected by the cold-room treatment.

Similarly, no observable gross or microscopic effects were produced in the adrenal of either the male or the female by exposure of the animal to ultra-violet radiation.

DISCUSSION

The present investigation has shown that exposure of either males or females of the hibernating rodent *C. tridecemlineatus* to extended and continuous low environmental temperatures failed to maintain the thyroid gland in a state of hyperactivity, in spite of the fact that it prevented sexual retrogression. Although at the time of autopsy the reproductive organs of the experimental animals exhibited a high degree of anatomical development, the thyroids presented evidence of low activity characteristic of the thyroid of the ground squirrel in hibernation. Apparently, the well-developed, potentially functional gonads failed to influence the thyroid when the environmental conditions had an inhibiting effect upon thyroid activity.

In the rat exposure to cold has been reported to have the opposite effect. It caused an outburst of heightened activity in the thyroid gland. The epithelium increased in height, and the colloid became liquefied and tended to disappear from the intrafollicular lumina, apparently being resorbed into the blood stream (Kenyon, 1933; Fischborn, 1935; Baillif, 1937). We must, however, bear in mind that in the case of the ground squirrel we deal with an animal upon which, by a special evolutionary adaptation, lower environmental temperatures have a physiologically inhibitory influence.

These results are compatible with the writers' previous conclusions regarding the thyroid-gonad interrelationships in the ground squirrel under investigation. The senior writer (1935) in a seasonal study of the *Citellus* thyroid has shown that, whereas heightened activity is generally observed in the thyroid gland of both the male and the female during the breeding season, this hyperactive phase in the annual thyroid cycle, characterized by high follicular epithelium and increased colloid absorption, persists throughout the summer and early autumn, i.e., throughout the animal's nonhibernating period. Therefore, it is not necessarily a result of gonadal influence but may be a phenomenon of metabolic origin. Furthermore, evidence was then presented to the effect that either

administration of male or female sex hormones or castration failed in both sexes to affect the thyroid gland, either grossly or microscopically.

In the adrenal gland, prolongation of high sexual development by means of low environmental temperature tends to stimulate high cortical development and differentiation of the reticular zone. This observation is in complete accord with results of a previous study (Zalesky, 1934), which show that a similar adrenal hypertrophy and differentiation occur in the normal reproductive cycle of both sexes during the period of breeding capacity, as well as following administration of gonadal stimulants or homologous sex hormones.

Thus we see that certain physiological processes in the adrenal cortex of the hibernating mammal under investigation are decidedly correlated with the animal's reproductive activities. The anatomical evidence of these processes has been described. Their significance, however, in the endocrine complex underlying reproductive activities will not have been determined until several additional phases of the adrenal-sex relationship have been cleared up. In that direction our investigation is now being further pressed.

SUMMARY

Twenty-four male and 13 female ground squirrels, *C. tridecemlineatus*, were individually caged at a temperature of $+4^{\circ}\text{C}$. Twenty-five males and 13 females, captured in the field at the same time as the experimental animals, were kept at room temperature as controls. The experiments were begun in April and early May, i.e., during the period of breeding activity and high sexual development. All animals in the cold room maintained their high sexual development, while all controls, on the other hand, regressed sexually within several months to a practically infantile condition.

Neither the low environmental temperature nor the prolongation of the high sexual development under the influence of low temperature seemed to affect, in either sex, the degree of physiological activity of the thyroid follicle as judged from its histological appearance. At autopsy in the autumn and winter the experimental animals, as well as the controls, yielded thyroids of the "resting" or "storing" type with little or no colloid resorption, low or cuboidal follicular epithelium, and abundant, usually dense colloid.

The adrenal gland of the experimental animals showed, at autopsy, a more highly developed and differentiated cortex than that of the control animals and approximated the cell picture normally seen in the adrenal cortex of the ground squirrel during the breeding period.

The adrenal medulla did not appear to be visibly affected in either sex by the cold-room treatment.

Exposure for 4 hours daily to ultra-violet radiation in addition to the cold-room treatment apparently did not affect the thyroid or adrenal glands.

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GROWTH OF PROTOZOAN CULTURES. II. LEUCOPHRYS PATULA AND GLAUCOMA PYRIFORMIS IN A BACTERIA-FREE MEDIUM

(Six figures)

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THE growth of a population of organisms is usually influenced by so many different environmental and other factors that true analysis of the resultant population changes is impossible, and one can do little more than fit empirical curves to the data. The purpose of this study is to investigate a simple population in a limited environment and to attempt in this special case so to control the conditions of experimentation that the interaction of factors responsible for rate of growth can be determined. For this work protozoan populations seem most suitable, but even the majority of these present great difficulties in control, since the food is bacteria. Some protozoa, though, can be grown in a bacteria-free medium and grown through a sufficient range of densities so that the phases of growth are distinctly separated. Results of some studies on such a population are reported in this paper.

THE PROTOZOA AND THEIR CULTURE

Leucophrys patula and *Glaucoma pyriformis*² are rather closely related holotrichous ciliates of the family Frontoniidae. The cultures of these organisms used in this growth study are from California, the *Leucophrys* (*L.*) isolated in 1934 by W. H. Furgason from near Stanford University and the *Glaucoma* (*G.*) in 1930 by J. O. Thomas from near Monterey. Both organisms were freed of all bacteria by individual washing (modified Parpart [1928] technique) and migration through dilute agar (0.5 per cent or less agar-agar). The *G.* were then cultured in 0.2 per cent Difco yeast extract, and the *L.* on the *G.* culture. Preliminary tests showed that *Colpoda duodenaria* was also suitable as food for the *L.* and that *Chilomonas paramecium* was unsuitable. Though culture of the very closely related *Colpoda steinii* under sterile conditions has been described by Oehler (1919), no successful culture medium was found in experiments on bacteria-free culture of *C. duodenaria*, and hence this protozoön was not used for further experiments. The *Colpoda* cultures for these preliminary tests were kindly given to me by A. G. R. Strickland, and the attempts at bacteria-free culture of *Colpoda* were also made by Strickland.

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² This organism is the same as the *Colpidium campylum* used by Taylor, Thomas, and Brown (1933), as the *Glaucoma pyriformis* used by Phelps (1935 and 1936), and as the *Tetrahymena glaucomaformia* described by Furgason (1936) and used by Giese (1938). A paper describing this organism in detail is to be published by Furgason (Arch. f. Protistenk., in press).

CULTURE CHAMBER AND COUNTING TECHNIQUE

Experimental cultures were grown in half-filled, 1-liter Florence flasks plugged with cotton. Inoculation and sampling were achieved through use of a U-shaped, 5-mm. glass tube and test tubes to which 4-mm. arms, plugged with cotton, have been attached as shown in Figure 1. The short test tubes were used for inoculation, and the longer ones for removal of samples. Control of the inoculation and sampling was by means of a rubber tube from the side arm to the operator's mouth. Care was always taken before withdrawing samples to mix the organisms thoroughly by shaking the flask and blowing back the liquid that was in the lower part of the 5-mm. tube.

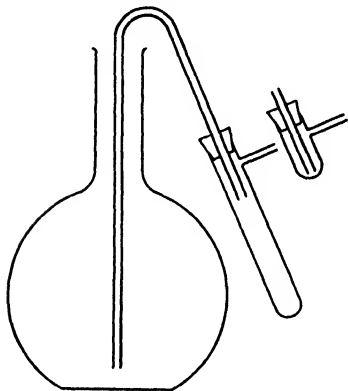


FIG. 1. -- Culture flask for study of protozoan populations.

After removal of about 2 ml. to the test tube and replacement of this tube by another similar, sterile one, samples were removed from the test tube with calibrated pipettes and placed in series of small drops on a glass plate. It was found advisable in using the micropipette always to empty it completely, since the last drop contains an excessively large number of organisms, indicating that some of the protozoa probably adhere to the glass until stripped off by the surface film.³ The number of organisms of each species in each drop on the glass plate was then counted; if there were too many protozoa in a drop for an accurate count of the swimming organisms, swimming was stopped by blowing a puff of air containing acetic acid fumes onto the drop, and then the killed organisms were recounted at a higher magnification.

EXPERIMENTAL DATA AND ITS ANALYSIS

A test run of growth of *G* alone at 25° C., using the above technique, is shown in Figure 2; the curve is similar to those obtained by Phelps (1935 and 1936). In this experiment the growth-rate in the exponential phase is 6.1 generations per day as compared with 5.6 generations per day reported by Phelps (1935), 6.3 generations per day reported by Phelps (1936), and 6.36 generations per day reported by Hetherington (1936), all tests made with Difco yeast extract as the culture medium and at a temperature of 25° C.

In all plots of growth curves in this report the theoretical curves for unlimited growth are represented by continuous lines, and the deviations due to limitations within the organism (lag phase of adjustment to the environment) and imposed upon the organism by the environment (phase of negative acceleration of growth) have been shown by broken lines drawn freehand.

The theoretical curves for the double culture are determined by the conditions that the organisms divide to produce two daughters at regular intervals and that the rate of

³ Phelps (1935) had found that the *G* would stick to the wall of a capillary tube through which a culture was flowing; in his 1936 work he eliminated any error due to organisms sticking to his pipettes by killing the *G* before pipetting and counting. Hetherington (1936) also uses a technique designed to overcome errors caused by adherence of *G* to the walls of his pipettes.

capture of G by L is constant. Under these conditions the equations for rates of growth are

$$\frac{dN_L}{dt} = r_L \cdot \ln 2 \cdot N_G \quad (1)$$

and

$$\frac{dN_G}{dt} = r_G \cdot \ln 2 \cdot N_G - r_c N_L, \quad (2)$$

where r_L and r_G are the division rates for L and G in units of generations per unit time and r_c is the rate of capture of G by L in units of G captured per unit time per L . Upon

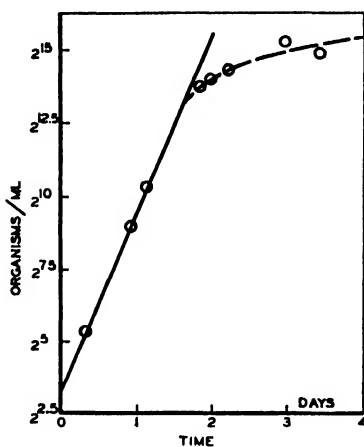


FIG. 2

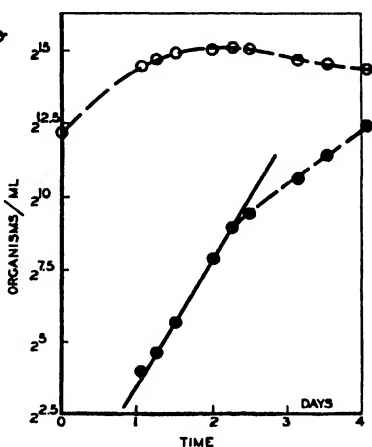


FIG. 3

FIG. 2.—Growth of *G. pyriformis* at 25° C. in 0.2 per cent Difco yeast extract

FIG. 3.—Growth of *L. patula*, ●, on *G. pyriformis*, ○, at 25° C. in 0.2 per cent Difco yeast extract

integration and introduction of the initial conditions that when $t = 0$, $N_L = (N_L)_0$ and $N_G = (N_G)_0$, equations (1) and (2) become

$$N_L = (N_L)_0 2^{r_L t}, \quad (3)$$

and

$$N_G = (N_G)_0 2^{r_G t} - \frac{r_c (N_L)_0}{(r_G - r_L) \ln 2} (2^{r_G t} - 2^{r_L t}). \quad (4)$$

In calculating the theoretical curve for N_G , the value of $r_G = 6.1$ generations per day from the data of Figure 2 has been used. The values of r_L and $(N_L)_0$ have been taken directly from the separate graphs of the growth of the L alone, then the experimental values for N_G and N_L at two times were introduced into equation (4) and the resulting simultaneous equations then solved for values of $(N_G)_0$ and r_c . For example, in the experiment plotted as Figure 4 the values of $r_L = 4.0$ generations per day and $(N_L)_0 = 14.9$ were taken from the data on growth of L alone, then the experimental data

$$\left\{ \begin{array}{l} t = 0.833 \text{ days} \\ N_L = 150 \\ N_G = 11,400 \end{array} \right\} \quad \text{and} \quad \left\{ \begin{array}{l} t = 1.416 \text{ days} \\ N_L = 740 \\ N_G = 3,800 \end{array} \right\}$$

were introduced into equation (4), and values of $(N_G)_0 = 1,710$ and $r_0 = 190 G$ captured per day per L were obtained. Points on the theoretical curve were then calculated, and it was found that the curve fitted the experimental data for N_G at $t = 0$ and $t = 0.2085$

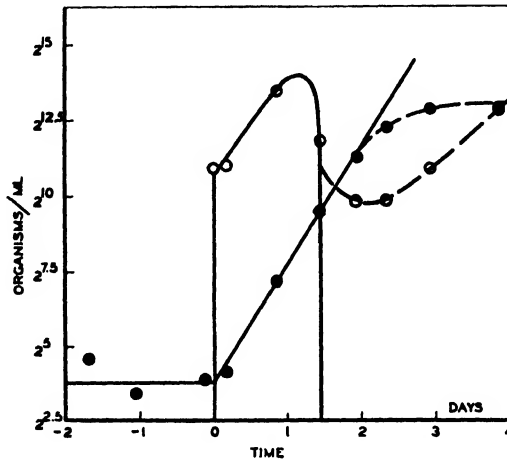


FIG. 4.—Growth of *L. patula*, ●, on culture of *G. pyriformis*, ○, at 25° C. Food organisms were absent in the culture flask until the *Glaucoma* were introduced at zero time.

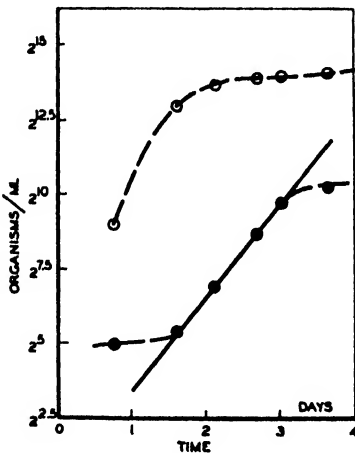


FIG. 5

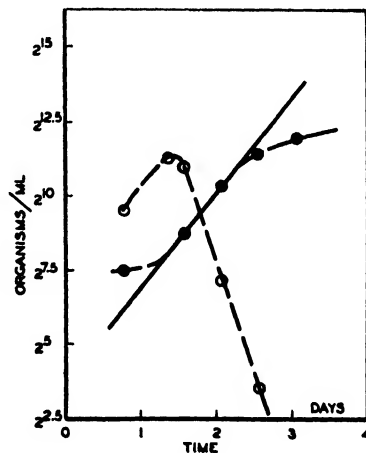


FIG. 6

FIG. 5.—Growth of *L. patula*, ●, on *G. pyriformis*, ○, at 25° C. in 0.2 per cent Difco yeast extract

FIG. 6.—Growth of *L. patula*, ●, on *G. pyriformis*, ○, at 25° C. in 0.2 per cent Difco yeast extract

days. Unfortunately, in most cases the conditions were such that the period throughout which both organisms were growing exponentially was too short for good determination of r_0 by this method. The value of r_0 in units of G captured per L between each fission is $r_0/r_L = 47$.

The mean value of r_L is 3.7 generations per day (Figs. 3, 4, 5, 6, and two experiments not shown by graphs). The values averaged for r_L , however, come from separate series of experiments in which the conditions were apparently not identical: values of 4.3, 4.2, and 4.0 were found in one series of experiments (e.g., Figs. 3 and 4); and values of 3.4, 3.3, and 3.0 were found in a later series (e.g., Figs. 5 and 6). Though there was no lag phase in any case in the first series and one present in each case in the second series, there is not thought to be any correlation between a lag phase and a lower fission rate in the subsequent exponential phase of growth.

DISCUSSION AND SOME QUALITATIVE OBSERVATIONS

Any representation of growth in the lag phase or the phase of negative acceleration of growth by theoretical curves seems quite inadvisable at present, and, indeed, for the phase of negative acceleration of growth it seems doubtful that the same formulation will fit cases in which different factors are limiting. However, these bacteria-free protozoan cultures seem very suitable for the experimental separation of factors involved in these phases, and through further experimentation it should be possible to examine the relations of environmental factors to phases of nonexponential growth and to give a sound basis for any exact mathematical formulation of growth in these phases.

Though quantitative analysis of phases other than those of exponential growth is not attempted, a number of qualitative observations should be noted. In the experiments shown as Figures 3 and 5 termination of exponential growth is apparently the result of factors due to high concentration of organisms, whereas in the experiments shown as Figures 4 and 6 termination of exponential growth is apparently caused by depletion of food organisms.

In Figures 3 and 5 it is seen that, though the concentration of G is such that they are in the phase of negative acceleration of growth, exponential growth of L is still found. One might suppose then that the L are less sensitive to low oxygen tensions than the G , since Phelps's studies seem to show that the limiting factor for growth of G under conditions similar to these is oxygen tension. However, since in several instances the G have been found to multiply after the L have entered a phase of negative acceleration of growth and since the L are larger than the G , it seems advisable to await direct measurement of growth under controlled conditions of oxygen tension before attempting an explanation of the nature of the intermediate factors terminating the exponential growth phase in these cultures in which concentration of organisms is a limiting factor.

In Figures 4 and 6 it is seen that exponential growth of L continues for a time after the rate of capture of G by L (r_c) is no longer constant but has decreased. This decrease in r_c is shown by the values of N_G lying above the extrapolation of the theoretical curves. The delay in decrease in r_L after r_c has decreased is most probably due to the presence of considerable food reserves in the undigested food vacuoles and in the protoplasm of the L which permit the continuation of regular fission without capture of many G .

It has also been observed that L introduced into fresh cultures show a lag phase, e.g., Figures 5 and 6, if taken from the phase of negative acceleration in cultures in which high concentration of organisms has become a limiting factor,⁴ but that there is no appreciable

⁴ Though the relation between duration of lag phase and position in the phase of negative acceleration of growth from which the organisms were taken has not been worked out, it was observed that organisms need not progress far into the phase of negative acceleration of growth before showing a definite lag phase, e.g., organisms taken from the experiment shown as Figure 4 at 2.2 days showed a very pronounced lag

lag phase if *L* are taken from the phase of negative acceleration or stationary state in cultures in which depletion of food organisms is the limiting factor, e.g., Figure 4, where, prior to zero time, growth is limited by absence of food organisms and where, upon introduction of *G*, the potential growth-rate for the environment is realized after little or no lag phase.

Further study of growth of *L* in the absence of food organisms showed that the *L* would maintain themselves for many days in the sterile Difco yeast-extract solution alone and even increase in number to a slight extent. It was noted that addition of yeast powder such as used by Hetherington (1936) improved growth in the absence of food organisms, and it has since been found by J. O. Thomas (personal communication) that the *L* will grow fairly well on the yeast autolysate used by Phelps (1936), without introducing any other organisms. Thus in these growth experiments the 47 *G* captured between each fission of *L* doubtless does not represent all the food obtained in this interval, since the experiments were carried out in a nutrient solution.

SUMMARY

1. Methods for culture and growth measurement are described for the double culture of *L. patula* and *G. pyriformis* in a bacteria-free medium.
2. Growth of *L. patula* is found to be exponential at a rate of 3.7 generations per day at 25° C. up to concentrations of the order of 2,000 per milliliter under the conditions of the experiments.
3. Analysis of the growth curves of *Glaucoma* and *Leucophrys* together to obtain the rate of capture of *Glaucoma* by *Leucophrys* is shown. It is found that approximately 50 *Glaucoma* are captured between each fission of *Leucophrys*.

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phase. This subsequent lag phase of organisms taken at 2.2 days in this experiment indicates that in this case concentration is a limiting factor, though both concentration and depletion of food are probably factors in this particular instance. That concentration was a limiting factor might also have been inferred from the later growth of *G* as seen in the figure, for in experiments such as that of Figure 6 in which low concentration of *G* becomes a limiting factor at a lower concentration of *L*, no *G* were found at later periods.

BIOCHEMICAL ASPECTS OF THE DIFFERENTIATION
OF THE FEMALE HONEYBEE
(*APIS MELLIFERA* L.)¹

(Seven figures)

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ALTHOUGH the dimorphism existing in the female honeybee (*Apis mellifera* L.) has claimed the attention of naturalists and biologists for centuries, little attempt has been made to study the physiology of the development of the two castes. The queens and workers are produced from identical larvae, as it is possible to rear queens from any female larvae by providing them with the proper food and care during early life. Furthermore, queens require between 12 and 13 days to complete their larval and pupal development after hatching from the egg whereas the workers complete their development in about 18 days. According to Snodgrass (1925), for the first two days after hatching all female larvae apparently receive the same diet. This diet is royal jelly, a secretion of the pharyngeal glands of the workers. During the third day the diet of the larvae that are to become workers is altered, but the queen caste continues to receive royal jelly. This difference appears to be a factor in bringing about the morphological and functional differences between the two female castes.

Melampy and Jones (1939) have shown that royal jelly from queen cells containing larvae between 3 and 4 days old has the following approximate chemical composition: moisture 66 per cent and dry matter 34 per cent, the latter consisting of protein 36 per cent, fat 16 per cent, total reducing substance (calculated as glucose) 37 per cent, ash 2.4 per cent, and undetermined material 8.6 per cent. Royal jelly is a good source of vitamin B₁, containing 1.0–1.5 I.U. per gram, but it contains no demonstrable amount of vitamins A or C.

The physiology of caste production in social insects is not clearly understood; however, several investigators have offered suggestions as to possible mechanisms involved. Huxley (1932, p. 62) reported that the workers and soldiers of the ants *Anomma nigricans* and *Camponotus gigas* represent a series of size forms of a single genetic type and "that the differences in size can only be supposed to be brought about by definite treatment of the larvae by their nurses, the largest types being fed to the limit, the smallest types being deprived of food, and so forced to pupate, while still quite small larvae." Castle (1934) has demonstrated that feeding alcohol and ether extracts of functional queens of the termite *Termopsis angusticollis* Hagen to isolated groups of fifth and sixth instars retards the development of female supplementary reproductives.

Hill and Burdett (1932) reported experiments from which they concluded that "the bees add vitamin E to royal jelly, on which queens are raised, and withhold it from

¹ The writers are indebted to Professor A. E. Emerson, of the University of Chicago, and Dr. M. Somogyi, of the Jewish Hospital, St. Louis, Missouri, for their helpful suggestions.

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worker larval food." Mason and Melampy (1936) assayed this secretion and found vitamin E to be absent, and their finding was confirmed by Schoorl (1936), by Evans, Emerson, and Eckert (1937), and by Haydak and Palmer (1938). Heyl (1939) has suggested the presence of a gonadotropic hormone in royal jelly because the injection of dilute sodium hydroxide and aqueous pyridine extracts of the secretion caused precocious development of the Graafian follicles in immature rats. However, this finding needs verification. In a recent review Emerson (1939) has suggested that the secretions and exudates consumed by social insects may contain specific substances (coenzymes) "which, when activated, can stimulate or inhibit development and behavior."

The work reported herein deals with the chemical changes associated with the development and differentiation of the two female castes, being part of a study of the physiology of reproduction in the honeybee. This dimorphism due to differences in nurture of individuals having the same gene complex furnishes an excellent example of the influence of environment on insect development.

MORPHOLOGICAL DIFFERENTIATION BETWEEN QUEEN AND WORKER

The queen and worker castes of the honeybee are adaptive to their specific function as imagoes and are not degenerate, each having many specialized characters. The queen is characterized by a short proboscis, notched mandibles, small brain, long sting, rudimentary pharyngeal glands, highly developed functional ovaries, and long life-span. On the other hand, the worker has a long proboscis, specialized mandibles, large brain, short sting, functional pharyngeal glands, undeveloped ovaries, and a comparatively short life-span. The primary function of the queen is reproduction, whereas the workers have more varied duties, such as collecting and storing nectar and pollen, secreting wax and building comb, providing protection, and rearing brood.

The mature queen honeybee weighs about 200-225 mg.; the writers obtained an average weight of 216.99 ± 1.63 mg. for 100 laying queens. The average weight of 2,425 workers they found to be 118.90 ± 0.26 mg.

The reproductive organs of the queen are highly developed and occupy a large part of the abdominal cavity. Eckert (1937) reported an average of 340 ovarioles in the ovary. Nelson (1924) noted that the rudiments of ovaries are much larger in the queen larvae than in the worker larvae, an indication that their development is greatly accelerated during later larval life. He also found their structure to be similar in the two castes except that in the queen larvae the presumptive rudiments of the ovarian tubules are both longer and more numerous. Oertel (1930) observed that in the worker larvae the number of ovarian tubules in each ovary decreases to 12-15 approximately 30 hours after sealing and that only 3 or 4 remain 120 hours after sealing. No further changes were observed in the workers 240 hours after sealing. Hambleton (1928) found a rudimentary bursa copulatrix, spermatheca, and spermathecal gland connected with the vagina in the adult worker honeybee. Mature eggs are sometimes produced by workers.

EXPERIMENTAL PROCEDURE

REARING AND PREPARATION OF INSECTS

The method for rearing the honeybees used in this work has been described by Melampy and Willis (1939). The larvae were removed from the queen cells or brood frames for analysis, washed with tap water, rinsed with distilled water, and placed between

sheets of filter paper to remove excess water. The guts were not removed since it is difficult to remove completely the alimentary tract containing the undigested food of the immature honeybee. There is no connection between the mid-gut and the hind-gut until 5-10 hours after the larva is sealed, according to Oertel (1930). At this time the waste material in the mid-gut passes to the hind-gut, and 20 hours after sealing it is entirely evacuated. Since the pupae and newly emerged adults were free from adhering material, it was not necessary to wash them.

ANALYTICAL METHODS

Growth, moisture, dry matter, nitrogen, and total lipid were determined at 24-hour intervals, whereas the total reducing substances, calorific value, and ash content were determined at 3-day intervals. Data from chemical analyses used in constructing Figures 2-7 represent averages of 4-10 determinations made over a period of 6 months. The amount of insect tissue used for the various chemical determinations varied with the stage of development of larvae and pupae.

Growth.—The growth data were obtained by weighing representative lots of insects at 24-hour intervals during the life-cycle. The insects were weighed in groups of 15-200 individuals, depending upon the stage of development. The average values used in plotting the growth curves represent the average weights of at least 200 individuals and not more than 1,200.

Moisture, total nitrogen, and ash.—The moisture determinations were made in a vacuum oven at 60° C. The total nitrogen was determined by the standard Kjeldahl procedure and represents tissue and chitin nitrogen as well as end products of nitrogen catabolism such as uric acid. Ash determinations were made in a muffle furnace at about 600° C.

Total lipid.—The total lipid was determined by the Kumagawa and Suto procedure as used by Slifer (1930). Two or three grams of living insects was dried *in vacuo* at 60° C. The dried tissue was pulverized and transferred to a tall beaker for saponification. A mixture of 25 cc. of 25 per cent sodium hydroxide and 5-10 cc. of ethyl alcohol was added, the beaker covered with a watch glass, and the whole heated for 3 hours on an electric plate. It was necessary to maintain a low temperature and to stir the mixture occasionally. The reaction was allowed to continue overnight at room temperature. The soap solution was then transferred quantitatively to a glass-stoppered bottle, and the fatty acids were liberated by adding 25 cc. of concentrated hydrochloric acid. After the mixture had cooled to room temperature, 40 cc. of pure ethyl ether was added. The bottle was shaken thoroughly and allowed to stand until the ether and aqueous layers had separated, when the ether layer, containing the lipids, was drawn off into an Erlenmeyer flask. The extraction was repeated six times, 25-cc. portions of ether being used, and the combined extracts were then evaporated at 35° C. The lipid residue was taken up in purified petroleum ether. The petroleum-ether extract and the subsequent petroleum-ether washings were filtered through fat-free filter paper into weighed small beakers, and the ether was allowed to evaporate at room temperature. The fat in the beakers was dried for 1 hour at 60° C. and cooled in a desiccator before being weighed. The total lipid represents the fatty acids as well as such ether-soluble, nonsaponifiable substances as the sterols.

Total reducing substances.—The total carbohydrate content of the tissue was deter-

mined in terms of total reducing substances calculated as glucose. It is believed that the amount of total reducing substances is an index of the carbohydrate content, because after fermentation with washed yeast the filtrates produced little, if any, color with the sugar reagent. This test demonstrates the absence of interfering substances, according to Somogyi (1928).

The macerated tissue was placed in a 50-cc. centrifuge tube, and 10 cc. of 1.2 N sulphuric acid added for each gram of tissue. The tube was closed with a rubber stopper, which was provided with a 2-foot glass tube serving as a reflux condenser. The tubes and their contents were heated for 3 hours in a boiling-water bath. When the digests were cool, they were neutralized with a saturated solution of barium hydroxide, phenolphthalein being used as an indicator, diluted to 50 cc., and filtered. The amount of total reducing substances was determined in the filtrate by the dinitrosalicylic acid method of Sumner (1924).

Calorific value.—The calorific value of the moisture-free tissue of queens and workers was determined at various stages of the life-cycle in order that energy changes accompanying the development of the two castes might be followed. An adiabatic calorimeter was used for this purpose, and the procedure was that described by Long (1934).

RESULTS

The results of these analyses on queen and worker honeybees are summarized in Table 1 and presented graphically in Figures 1-7.

TABLE 1
AVERAGE WEIGHT, CHEMICAL COMPOSITION, AND CALORIFIC VALUE OF THE
DEVELOPING WORKER AND QUEEN HONEYBEE

Age (Days)	Live Weight (Mg.)	Water (Mg.)	Dry Weight (Mg.)	Nitrogen (Mg.)	Lipid (Mg.)	Reducing Substances (Calcu- lated as Glucose) (Mg.)	Ash (Mg.)	Calorific Value (Cal.)
Worker								
3-4.....	25.38	21.07	4.31	0.54	0.57	0.77	0.27	23
6-7.....	136.80	103.78	33.11	2.09	5.37	12.07	1.00	177
9-10.....	130.05	102.36	28.29	1.92	5.04	8.51	1.03	161
12-13.....	131.31	105.03	26.28	2.11	4.62	8.27	1.01	145
15-16.....	126.12	105.45	20.67	2.03	2.84	3.30	0.99	110
18-19.....	113.36	92.94	20.42	2.18	1.44	0.88	1.04	104
Queen								
3-4.....	44.93	36.58	8.35	0.73	1.43	0.99	51
6-7.....	202.34	197.62	64.72	5.72	12.84	13.17	0.21	425
9-10.....	242.05	189.09	52.96	5.10	10.44	9.03	0.24	318
12-13.....	195.54	155.47	40.07	4.96	3.91	3.48	0.21	214

DISCUSSION OF RESULTS

Growth.—The growth curves in Figure 1 are semilogarithmic representations of increase in mass per individual insect plotted against age. They demonstrate that the two castes develop at approximately the same rate during early larval life, after which the growth of the worker is retarded, and the time necessary for development is extended beyond that required by the queen. The weight of a newly hatched larva is approximately 0.1 mg., and the maximum live weight attained by the queen during development is more than 2,500 times greater. On the other hand, the worker increases about 1,400 times. Elser (1929) reported a 7-day-old queen weighing nearly 300 mg.

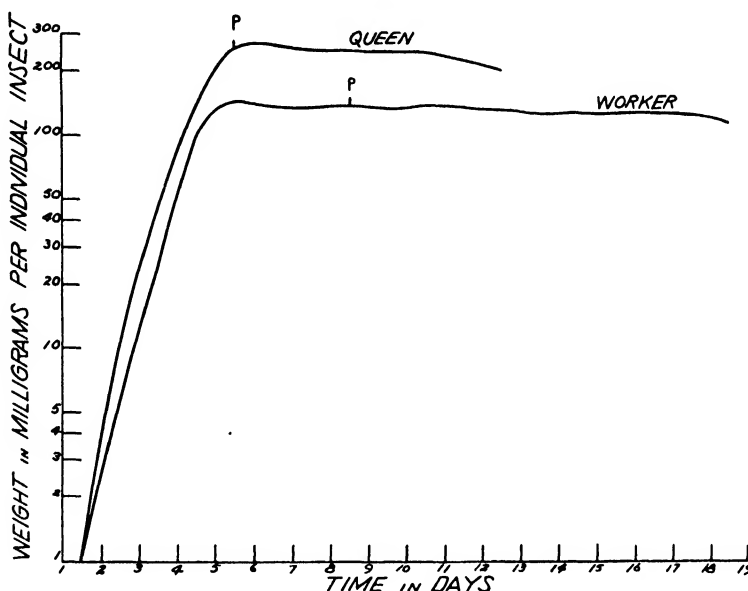


FIG. 1.—Growth curves for the queen and worker honeybees. P indicates the time of pupation

The growth curve for the worker is in agreement with the curve for the same caste reported by Nelson, Sturtevant, and Lineburg (1924).

Dry matter and moisture.—The highest percentage of dry matter and the lowest percentage of water occur at about the 5–6-day stage for the worker and the 6–7-day stage for the queen (Fig. 2). However, the absolute amounts of dry matter and water per individual insect (Fig. 3) are both at a maximum at these periods. The queen and worker parallel each other in percentages of dry matter and water, although the former develops at a greater rate.

It is suggested that the increase in dry matter for both castes during the larval stage is due to the formation of tissues, organs, and reserve materials. At this time the anabolic processes predominate over the catabolic; hence the observed increment in dry matter (Figs. 2 and 3, Table 1) is the difference between the amounts synthesized and the amounts utilized.

During the pupal period there is a gradual decrease in the percentage of dry matter for both queens and workers, which may be accounted for by the utilization of reserves, spinning of the cocoon, and evacuation of the gut.

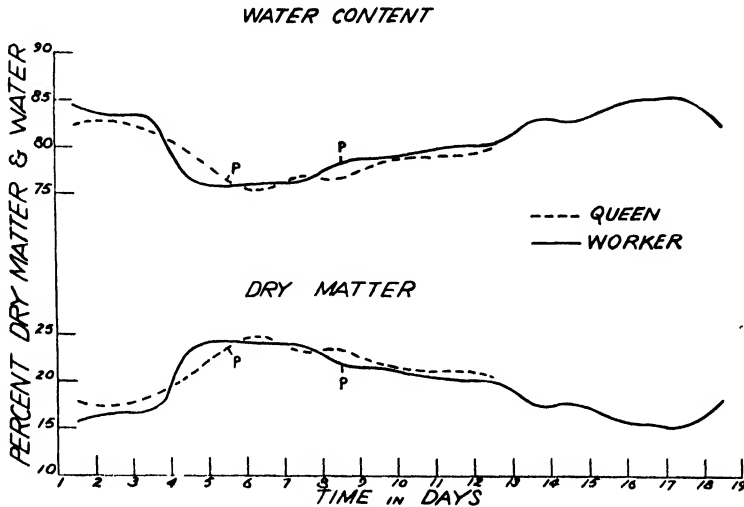


FIG. 2. Percentages of dry matter and water in the queen and worker honeybees during development. *P* indicates the time of pupation.

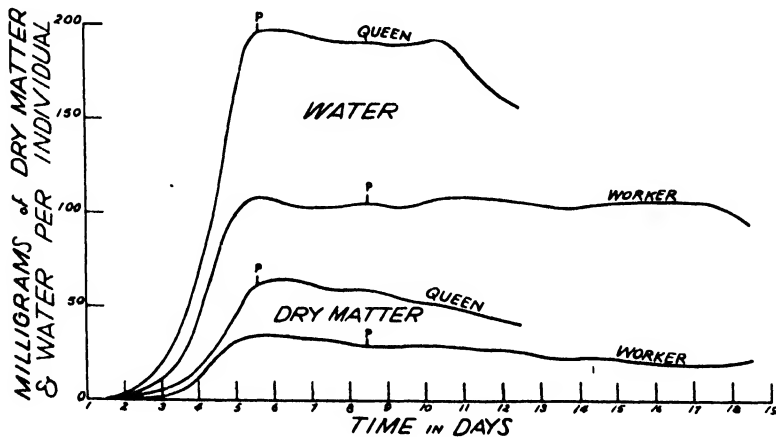


FIG. 3.—Dry matter and water content in milligrams per individual queen and worker honeybees during development. *P* indicates the time of pupation.

The gradual increase in the percentage of water during pupation is a result of a nearly constant amount of water per individual insect and a decreasing total live weight. The decrease in the total weight is due principally to the utilization of energy reserves, which constitute a significant amount of the dry matter at the beginning of pupation.

However, just prior to emergence there is a drop in the water content per individual insect.

Kellner, Sako, and Sawano (1884) found 1,395 gm. of water and 374 gm. of dry matter in 1,000 silkworm (*Bombyx mori* L.) larvae, but only 830 gm. of water and 339 gm. of dry matter in the pupae, demonstrating that the loss in weight during metamorphosis for this species is due largely to a loss of water.

Water is the most abundant constituent of the developing honeybee, comprising from 76 to 85 per cent of the total live weight of the worker and from 75 to 83 per cent of the queen. The water contained within the insect body is either ingested with the

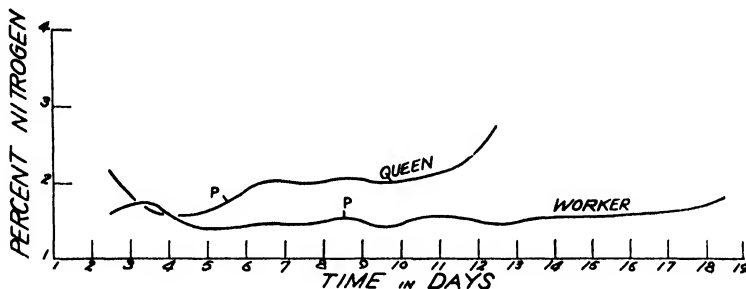


FIG. 4.—Percentages of nitrogen in the queen and worker honeybees during development. *P* indicates the time of pupation.

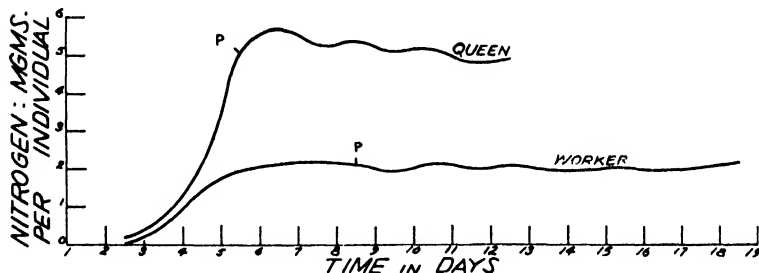


FIG. 5.—Nitrogen content in milligrams per individual queen and worker honeybees during development. *P* indicates the time of pupation.

food or produced within the cells during metabolism. The oxidation of fat and carbohydrate during pupation furnishes energy for adult morphogenesis and simultaneously produces water for cellular functions. The quantity of metabolic water produced by this method depends upon the rate of oxidation and the percentage of hydrogen in the metabolites being consumed. Metabolic water is also produced during growth by dehydrating processes, such as the formation of glycogen from glucose and of structural proteins from amino acids. Metabolic water is of physiological importance to the honeybee during pupation, since no water is ingested during this period.

Nitrogen content.—The percentage of nitrogen (Fig. 4) shows a drop between the second and fifth days of larval life, whereas the actual amount of nitrogen (Fig. 5) shows an increase. During this interval total reducing substances (Table 1), total lipid

(Figs. 6 and 7), water content (Fig. 3), and ash (Table 1) are increasing; hence the decrease in the percentage of nitrogen. During the pupal stage there is an increase in the percentage of nitrogen, and this increment is greater for the queen. By this time energy reserves have been depleted, and therefore the nitrogenous constituents are relatively more abundant.

The nitrogenous substances required for histogenesis by the embryonic cells of the imaginal buds are probably furnished by histolysis of the larval structural tissues; hence the quantity of nitrogen remains relatively constant during pupation and adult morphogenesis. Inouye (1912), Evans (1932), and others have reported that the total nitrogen content of other species of insects is constant during pupation.

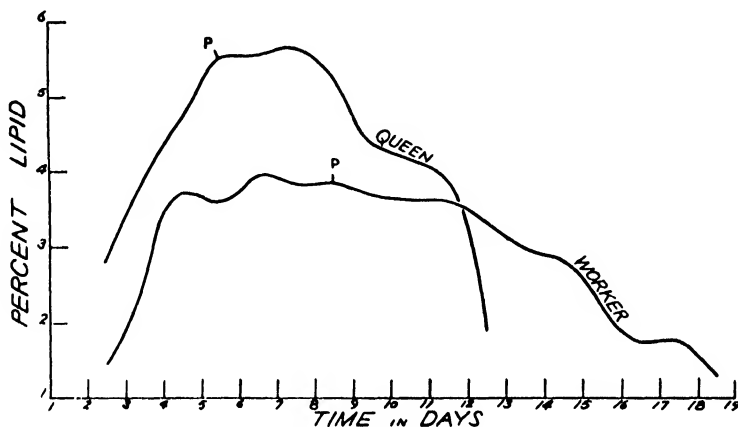


FIG. 6. Percentages of total lipid in the queen and worker honeybees during development. P indicates the time of pupation.

Differences in weight of queens and workers are shown to be due in part to differences in the quantity of nitrogenous constituents. For example, queens 6-7 days old have more than twice as much nitrogen as workers of the same age.

During the larval period there is a marked increase in lipid content which is probably synthesized from dietary fat and carbohydrate. That fatty materials are important in the development of this insect is suggested by their rapid disappearance during metamorphosis (Figs. 6 and 7). This observation is in agreement with the work of Straub (1911). Rudolfs (1926), working with the eastern tent caterpillar (*Malacosoma americana* [F.]), found that the ether-soluble extract increases during the larval period and the first part of metamorphosis and decreases later. Evans (1932) and Yuill and Craig (1937) reported a decrease in the amount of unsaturated fatty acids during the transformation of *Lucilia sericata* (Meig.).

Ditman (1938), working with the corn ear worm (*Heliothis obsoleta* [Fab.]), has shown an increase in the actual fat content and the percentage of fat on a dry-weight basis during the prepupal period. He indicated that there is a conversion of glycogen to fat by the corn ear worm during this period.

Total reducing substances.—The data for total reducing substances indicate a storage of carbohydrate during the larval period, which is utilized in pupal development, as

both queens and workers emerge with a small amount of reducing substances. Between the first and second 3-day periods the queens increase their total reducing substances thirteen times and the workers nearly sixteen times. The storage and utilization of total reducing substances parallel lipogenesis and lipid utilization (Figs. 6 and 7).

Ronzoni and Bishop (1929) have shown that the substances produced by the larval honeybee during carbohydrate digestion enter the blood and are partly converted and stored as fat and glycogen and partly remain as free and bound sugar in the blood. Straus (1911) reported that toward the end of the larval period more than 30 per cent of the dry weight is glycogen, whereas Parhon (1909) found that the adults contain 0.14-0.21 per cent of glycogen.

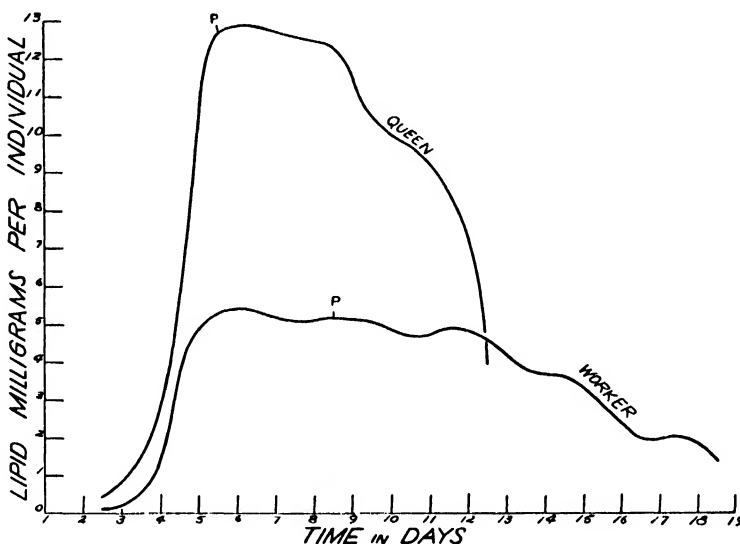


FIG. 7.—Total lipid content in milligrams per individual queen and worker honeybees during development. P indicates the time of pupation.

Bataillon (1893), studying the carbohydrate metabolism of the silkworm, found that the quantity of glycogen nearly doubles during the period of spinning and molting but that near the end of pupation nearly all the glycogen reserve has been utilized. Snodgrass (1925, p. 218) suggested that the cells of the fat body function as depots for glycogen reserves. If a mature larva is cut open and placed in iodine, the presence of glycogen in the exposed tissue of the fat body is indicated. Glycogen is found in the liver of higher animals but is also present in the muscles and in other tissues.

Crescitelli and Taylor (1935) found an increasing concentration of reducing substances in the wax moth (*Galleria mellonella* [L.]) during the period of spinning. This finding is in agreement with the results of Bataillon (1893) with the silkworm, Frew (1929) with the blowfly, and Ludwig (1932) with the Japanese beetle (*Popillia japonica* Newm.). Crescitelli and Taylor demonstrated that during the pupal stage the concentration of reducing substances decreases at first and then rises, and they suggested that, if the reducing substances consist mainly of glucose, a synthesis of this sugar may occur

during metamorphosis of the wax moth. Further citations on carbohydrate metabolism of insects are given by Uvarov (1928).

Ash content.—The workers contain more ash than the queens at all ages. The difference in ash content of the food probably accounts for the difference in the amounts of inorganic constituents found in the two castes. Royal jelly contains approximately 0.82 per cent of ash, whereas pollen contains 3 per cent or more.

Calorific value.—The calorific value of the tissue increases during the larval period, and there is a considerable expenditure of energy during metamorphosis. The calorific data confirm the changes already presented for dry matter, lipid, and total reducing substances. The reduction in calorific value during pupation is the result of the decrease in the total amount of fat and carbohydrate and, possibly, the accumulation of the end products of protein catabolism. The application of the calorimetric method to insect physiology provides a method of measuring energy utilization by the insect during development.

SUMMARY

The differentiation of the female castes of the honeybee (*A. mellifera* L.) has been studied from the biochemical standpoint. The findings of this study show that the queens and workers have the same approximate growth-rate during early larval life, and then the growth of the worker caste is retarded, as shown by the nitrogen, total lipid, total reducing substances (calculated as glucose), and calorific value.

This generalization is supported by the following experimental results:

1. The queens attained a maximum live weight of more than 260 mg. during development, as compared with 144 mg. for the workers.
2. The water content comprised 76–85 per cent of the total live weight of the worker and 75–83 per cent of the queen during development. The percentage of water was lowest at about the 5–6-day stage for the worker and 6–7-day stage for the queen. During the pupal period the water content was nearly constant, owing to the production of metabolic water.
3. The queens reached a maximum in nitrogen content at 5.72 mg. per individual, and the workers at 2.18 mg.
4. The highest total lipid content of the queen was 12.84 mg. per individual, whereas that of the worker was 5.37 mg.
5. Total reducing substances reached a maximum of 13.17 mg. for the queen and 12.07 mg. for the worker.
6. The average ash content of the prepupal and pupal stages of the worker was approximately five times that of the queen.
7. The calorific value of the tissue of both castes increased during the larval period and decreased during metamorphosis.

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PATHS OF WATER EXCHANGE IN THE EARTHWORM¹

(Eight figures)

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GEGENBAUR (1853) first suggested the nephridia of the earthworm as excretory in function, enlarging on von Siebold's earlier observation of the expulsion of fluid from the nephridiopores. Overton (1904) noticed in manipulating worms an initial loss of weight which was attributed to loss of nephridial contents. No supporting evidence for the nephridia as the site of origin was found by Adolph (1927) or Maluf (1939), these latter authors holding that most of the weight losses which follow upon successive weighings and handlings of earthworms were from the gut; and it was demonstrated that water might be lost from the mouth as well as from the anus. Darwin (1881) showed that these animals practiced "extra-stomachal" digestion in which they voided fluid from the mouth. Wolf (1938) also observed the voiding of buccal fluid in the beginning phases of dehydration.

It was proposed in the present studies to estimate quantitatively the part played in the water exchanges of the earthworm, *Lumbricus terrestris* L., by both of these paths (gut and nephridia) and by the integument.

METHODS

Adult worms were stored in moist earth at temperatures ranging from 4° to 13° C. Before use they were transferred to a constant-temperature room at either 18° or 20° C. and allowed to remain in 3 to 6 mm. depth of glass-distilled water in covered glass vessels for at least one night. When being weighed, specimens were lifted from their containers with a blade of a pair of forceps introduced carefully under the body just behind the clitellum. The worm was disturbed as little as possible, drained for a uniform period, and weighed in a tared aluminum cup, after which it was poured back into its container. Worms which were "handled" before weighing were lifted out onto toweling paper, rolled three or four times with dry filter paper, and either weighed directly or else dipped in water so as to become wet, whence they were again removed, drained, and weighed.

Eleven preliminary measurements indicated that, on dipping, water to the extent of 2.68 ± 0.17 per cent of the body weight adhered to specimens which had been superficially dried by handling. In all cases the sign \pm indicates the standard error.

Three terms should be defined. "Handling" refers to the procedure of rolling a worm with filter paper to dry the surface or to induce the expulsion of fluid from the nephridia and/or gut. "Basal weight" is the weight an individual attained by the expulsion of its water stores such that continued handling induced no further appreciable fluid loss.

¹ The writer is indebted to Professor F. F. Adolph for the interest, encouragement, and constructive criticism he gave to make this work possible and to W. B. Latchford for making the capillary cannulas used.

"Gross weight" (with respect to water) is the weight an individual attained when lying in water for a time long enough that at any succeeding times its water stores did not significantly increase.

DIURNAL FLUCTUATIONS

Adolph and Adolph (1925) noted that earthworms in tap water maintained constant weight only approximately. Maluf (1939) attributed these "minor fluctuations" to kidney activity. The fluctuations of weight were investigated in the present work by

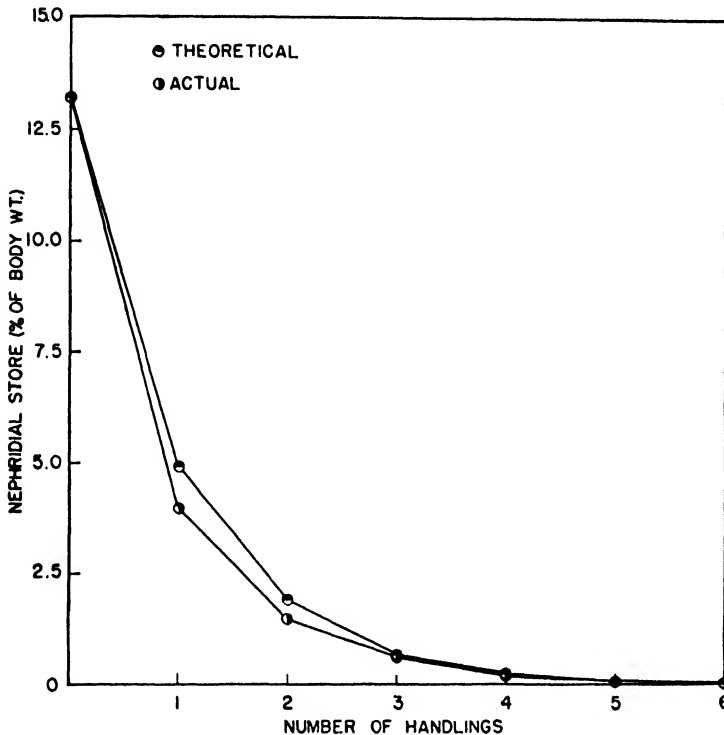


FIG. 1.--The effect of repeated handlings on nephridial water stores. The theoretical curve is plotted from the equation in text. Actual data do not significantly differ from this at any point.

studying the diurnal variations of weight of 12 individuals, each in a separate container of distilled water. Every 24 hours each was weighed to obtain the gross weight, while some of these were then handled to obtain the basal weight. Worms were handled until the difference between two successive weights was less than 1 per cent of the last weight recorded. The interval of time between handlings varied from 2 to 8 minutes; within this range the interval had no significance. The environing water was renewed daily.

Data on 32 worms reduced to basal weight showed that the amount lost per handling, regardless of the initial amount of fluid previously stored, was approximately a constant fraction of the amount in store (Fig. 1). The loss per handling was found to be 63.1 ± 2.4

per cent of the amount contained; and this held at least between stores of 23.5 per cent and 2.0 per cent of basal weight, though as basal weight was approached variation increased. From this the suggestion of Adolph (1927) that the loss is a logarithmic function of the number of handlings is confirmed and

$$L_n = L_i (1 - a)^n,$$

where L_n is the fraction of nephridial load after n handlings, L_i is the nephridial load, and a is a constant whose value under these conditions was 0.631 (63.1 per cent).

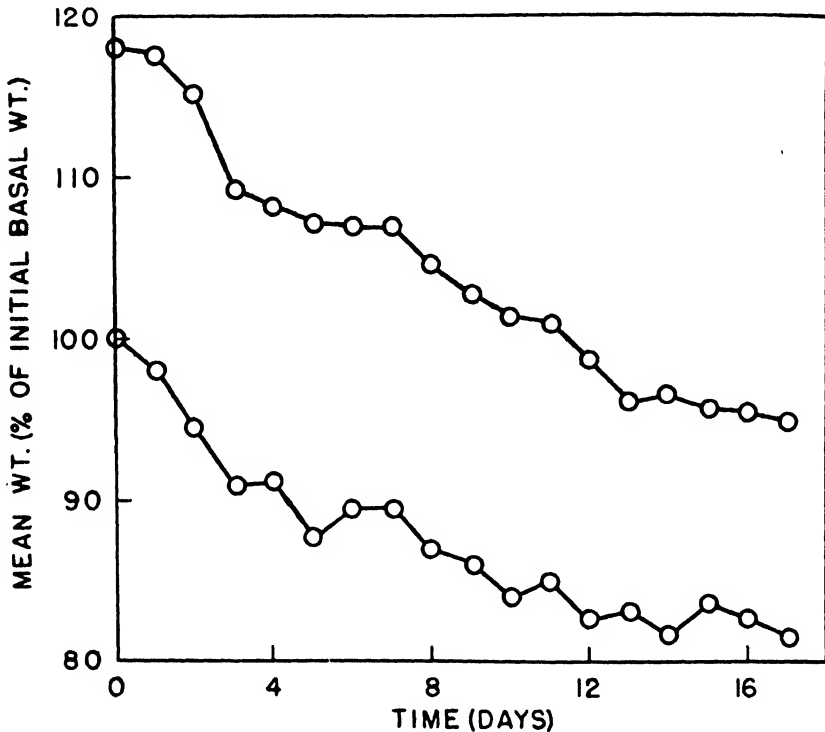


FIG. 2. Parallel downward trend of gross (upper curve) and basal (lower curve) weights at 17°-19° C. Data are corrected to offset the influence of dying worms on the average weight at a given day. Through the third day, 8 worms are averaged; through the fourth, 7; sixth, 5; twelfth, 4; thirteenth, 3; seventeenth, 2.

Figure 2 shows how gross and basal weights vary with time. The steady fall in weights of worms with time under these conditions is statistically significant. An undetermined but small part of the fall is probably due to defecation in worms whose guts contained some earth and to starvation. It is believed that loss of body fluid through dorsal pores in handling may also be a factor. Permanent loss of salts (accompanied by water) from the worms to distilled water may play a role.

Figure 3 indicates the weight changes of worms placed in a 0.01 M sodium chloride solution. The increase of water load under these conditions is marked. Data were also obtained on a single worm after substituting distilled water for the sodium chloride solution, whereupon loss of body weight followed. These data suggest that loss of salts and hence of water may account for some of the weight loss experienced by worms in distilled water. Maluf (1939) detected losses of chloride from worms only after they were ligated at both ends before their introduction to tap water.

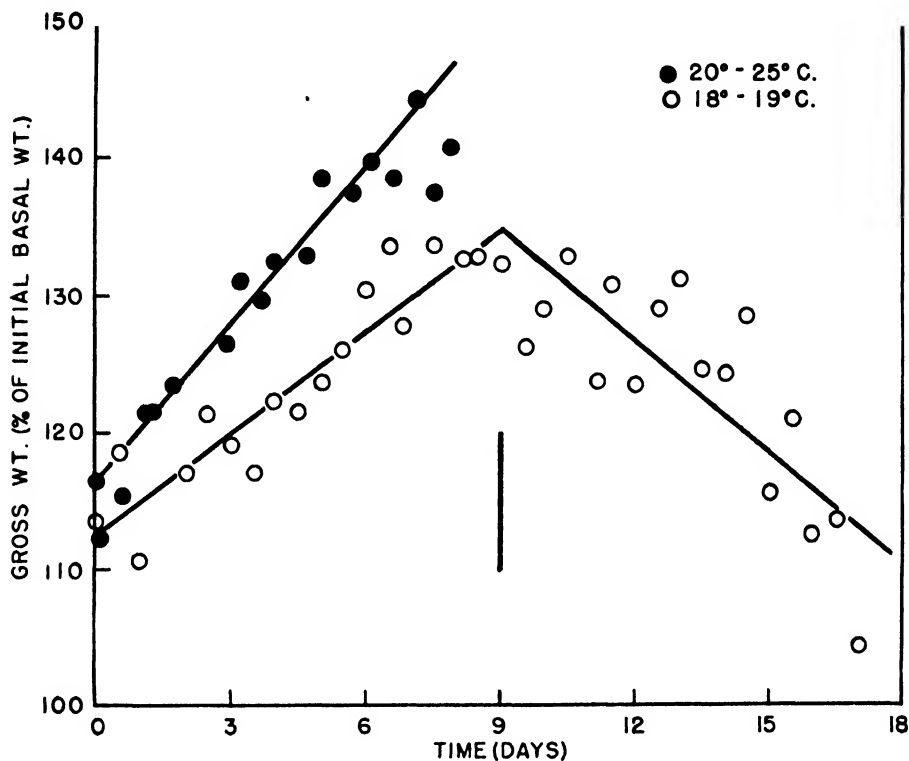


FIG. 3.—Solid circles represent the mean weights of 10 worms in 0.01 M NaCl solution. Open circles represent weight of one worm in 0.01 M NaCl solution until it was transferred on the ninth day to distilled water. The transfer is followed by an abrupt change of slope.

Not only is the fall of basal weight of interest but also the nature of the diurnal fluctuations of the basal and gross weights. The average difference of weight of worms from day to day in percentage of the initial basal weight was found (Fig. 2) to be 2.3 ± 0.3 per cent for the basal weights and 3.2 ± 0.3 per cent for the gross weights of the same worms. The difference is significant. When approximately as many more gross weights of specimens that happened to be available are included in the average difference for gross weights, the result is 3.4 ± 0.2 per cent. These statistics will be discussed in the next sections.

INTESTINAL OUTPUT

From a few data on the recovery of water losses sustained after handling, Adolph (1927) estimated that intake proceeded at a rate of approximately 0.8 per cent of the body weight per hour. Maluf (1939) estimated that a 15 per cent increase in weight

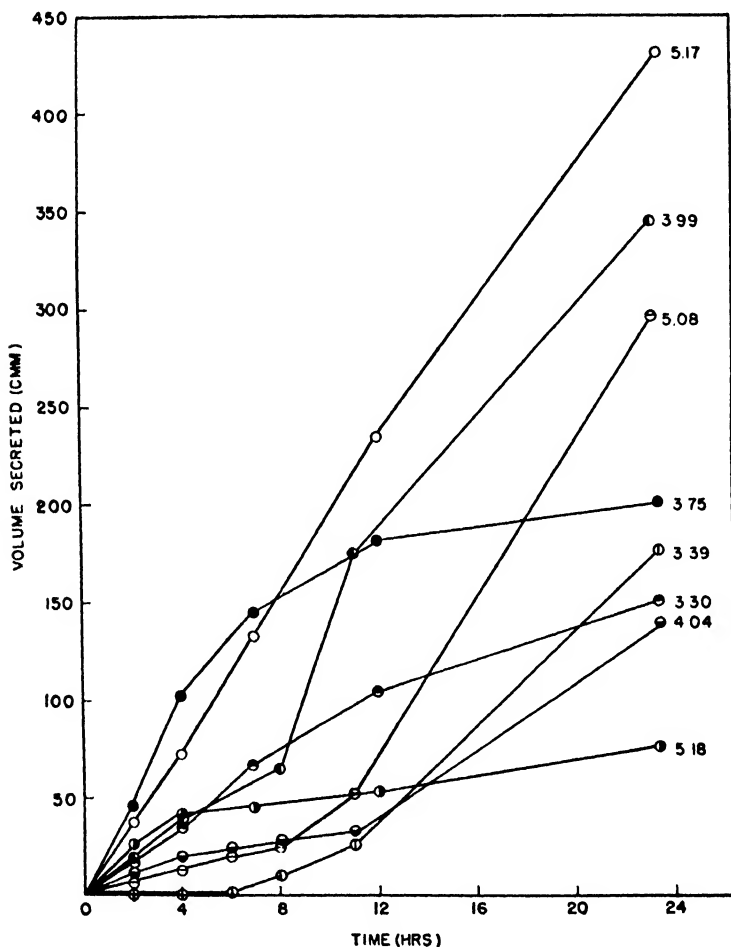


FIG. 4. --Fluid output of the gut. Rates were determined by using slope of curve between last two points. Temperature at zero time was 19°-20° C. but fell off with time to 15°-16° C. Gross weights of specimens are indicated in grams.

could occur in worms transferred from soil to tap water in about 12 hours. These data were taken to represent rates of filling of the gut, which apparently was able to lose 8.5 per cent of the body weight of the worm through successive handlings (Adolph, 1927) after it had remained undisturbed in water overnight.

However, it was found in the present experiments that, if both mouth and anus were

either ligated or stoppered with small glass plugs tied in place firmly with ligature thread (but not so tightly as to induce posterior autotomy) and a worm was allowed to come to gross weight overnight, it still lost an average of 13.4 ± 0.9 per cent of the basal body weight by handling. It is interesting to note that one individual was obtained which showed persistently high losses on handling, in one instance expelling 47 per cent of its basal weight at the time of handling.

It seemed that this loss of fluid was from the nephridiopores. This could be ascertained in anterior segments which possess no dorsal pores. On occasion too severe handling may have induced a *saignée* or draining reflex (Cuénot, 1898), whereby the worm suffered a loss of coelomic fluid from the dorsal pores locally, but this was not definitely established.

The contribution of the gut to water exchanges remained to be seen. In ordinary handling, little or no voiding of feces was observed. Voiding more often follows rough treatment. Lack of defecation was regarded as presumptive evidence of lack of appreciable voiding of water from the anus; and only minute quantities of fluid were observed to be cast from the mouth, this act also in part being dependent on the strenuousness of handling.

Jackson's (1925) histological studies on a single worm indicated that the intestinal lumen occupied about 15.2 per cent of the body volume. It is doubtful that ordinary handling could express such a volume of fluid, especially since the digestive tract is often laden with earth and mucus, undoubtedly requiring persistent peristalsis for their removal. No evidence for removal of such large amounts of feces was found.

With evidence for the nephridial path as a considerable factor in the water exchanges of the earthworm but with the existence also of a definite gut path, a quantitative estimation of the magnitude of each was attempted. To measure the excretion rate of the intestine, specially designed cannulas constructed of capillary tubing, calibrated for volume per unit length, were inserted into the mouth alone, while the anus was plugged (or vice versa); or one worm was allowed to excrete into a cannula at each end. The volume of fluid put out was thus determined at the level of the worm at intervals (Fig. 4). At 15° and 16° C. rates were found to be 0.22 ± 0.13 per cent of the mean gross weight per hour of 15 worms and bore no relation to body weight within the range of weights of worms used. A few manometric tests revealed that fluid was extruded into a mouth cannula until a maximal pressure of approximately 4.5 cm. of water was reached.

NEPHRIDIAL OUTPUT

Figure 5 shows the apparent rate of gain of weight, falling with the frequency of handlings. This is believed to be due to the loss of fluid discharged upon dropping the worm into the tared cup. Adolph (1927) showed that similar manipulation could induce the expulsion of fluid. While the weight of the worm and its nephridial contents may thus be obtained at any given weighing, the procedure robs the worm of a fraction of its nephridial content so that the next period starts from a smaller load than is indicated by the point on the graph. However, the value for α for this procedure may not be the same as that for handling. The slope of the line between the point representing basal weight (abscissa) and the point representing the next weighing might be used as a minimal value for the rate of gain of weight of an undisturbed individual; and this may be regarded also as the rate of excretion of fluid by the nephridia into their reservoirs.

The rate of excretion thus determined is not found to vary with the interval of time between the first two points, suggesting that until the reservoir is filled, the rate of

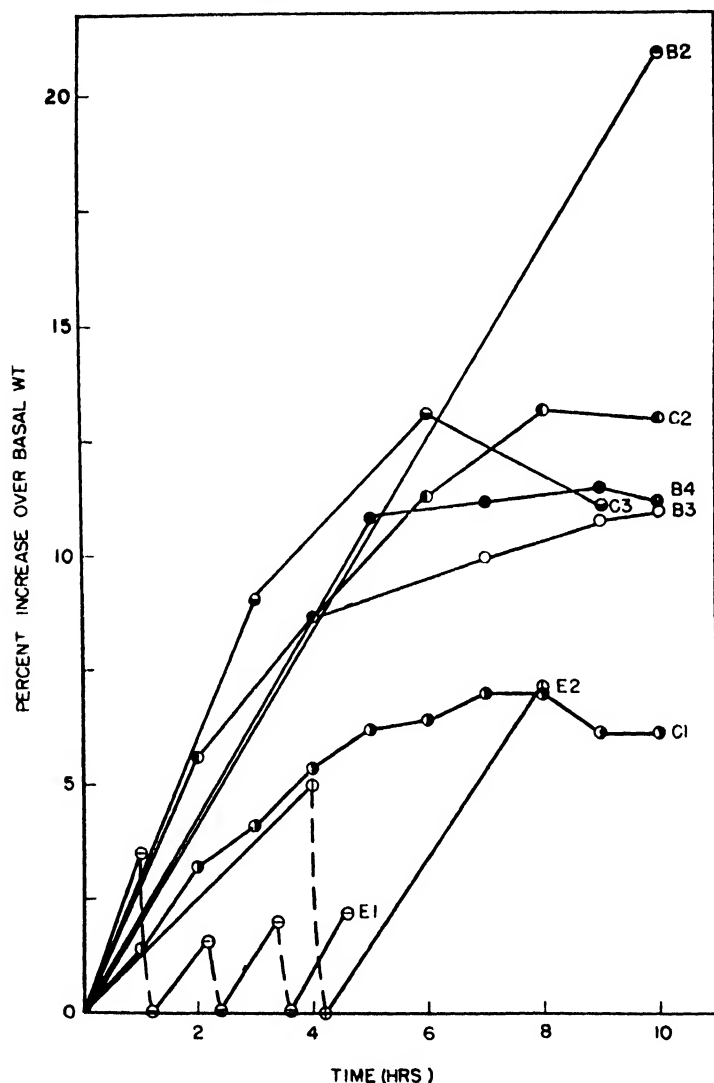


FIG. 5. - Intake rate of integument or excretion rate of nephridial battery may be determined from initial slopes. Broken lines indicate that the same individual was reduced to basal weight again. Worms are in water balance.

accumulation of fluid is constant. What happens when the reservoirs of undisturbed worms become filled has not been ascertained. Cuénot (1898) asserted that the reservoir is emptied about every 3 days in order that passage of coelomic fluid through the

excretory organ may be maintained, but there seems to be no firm basis for this view. The excretion rate might fall off, or there might be a periodic expulsion of fluid from the muscular duct. Such expulsions would conceivably play a part in giving rise to diurnal fluctuations.

In the above tests worms were weighed with adhering water and lifted carefully as described in the section on methods; it is likely that little initial expulsion of fluid is induced by this method of weighing.

The minimal rate of intake (and excretion) estimated from the initial slopes in 68 cases (on occasion the same worm was reduced to basal again after the first weighing and retested) averaged 2.55 ± 0.14 per cent of the basal weight per hour at 19° and 20° C. No significant differences were noted between worms having buccal and anal orifices either open or stoppered by plugs or ligation. In 41 cases where worms plugged or ligated at both ends were employed, the average rate was 2.49 ± 0.20 per cent of the basal weight per hour; and in 27 cases of unplugged specimens the rate was 2.84 ± 0.27 per cent. If a significant difference between the means of the two types of specimens had been manifested, it might be expected, as was actually found, that the plugged or ligated specimens would show lower rates, since tying off the extremities injures the region, probably eliminating the function of some nephridia, thus reducing the size of the effective battery of units.

THE INTEGUMENT

In worms in water balance the total rate of output is equal to the rate of excretion of the nephridia plus that of the intestine, the larger portion being the nephridial one. If worms are dehydrated to varying degrees by exposure to air, they may, on replacement in water, regain body weight at rates varying with the water deficit (Fig. 6). This shows the ability of the integument to take in water more rapidly than nephridia eliminate it.

The plotting of Figure 6 requires further explanation. If we wish to distinguish rates at which the integument takes up water after worms suffer varying water losses, it is most convenient to compare them early in the recovery period, since they ultimately fall off to the normal turnover rate as the worms return to water balance. It has been shown that the recovery of water by dehydrated, isolated muscles is proportional to the square root of time for initial periods (Adolph, 1931; Wolf, 1940; and others). It appears from a few data that this also holds for dehydrated earthworms (Fig. 7). For Figure 6 an arbitrary recovery time (49 min.) for dehydrated specimens was chosen, as well as an arbitrary degree of dehydration, so that complete recovery would not be attained in the given time, and most dehydrations permitted viability (Jackson, 1925).

So long as

$$I = k\sqrt{t}, \quad (1)$$

where I is the intake in percentage of basal weight in t minutes and k the constant of proportionality,

$$k = \frac{I}{\sqrt{t}} = \frac{I}{7}. \quad (2)$$

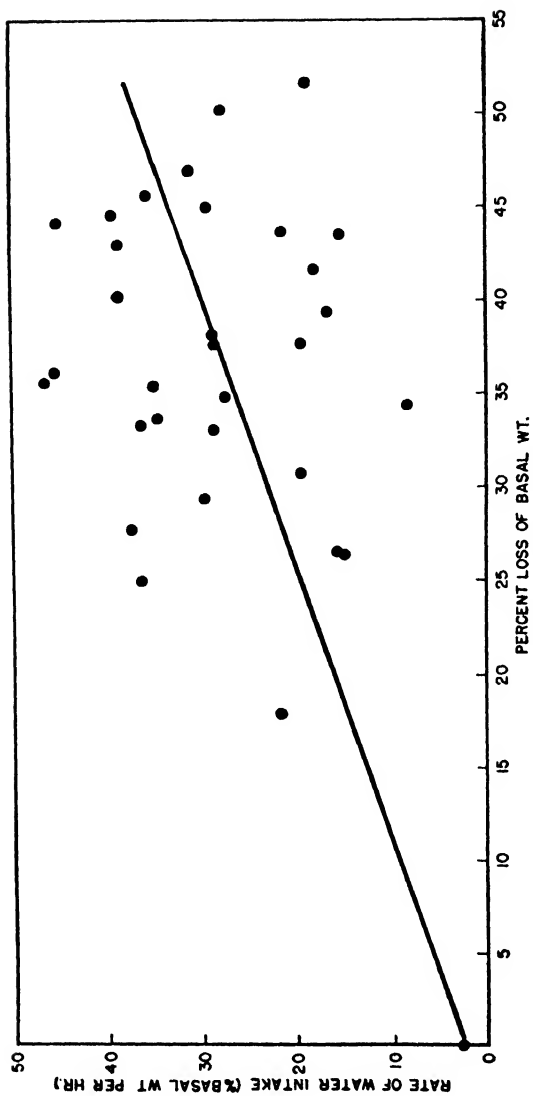


FIG. 6.—Net rate of intake of water through the integument at ninth minute of recovery. The point at zero per cent loss of weight represents the mean excretion rate of the nephridia determined in water balance from 68 cases.

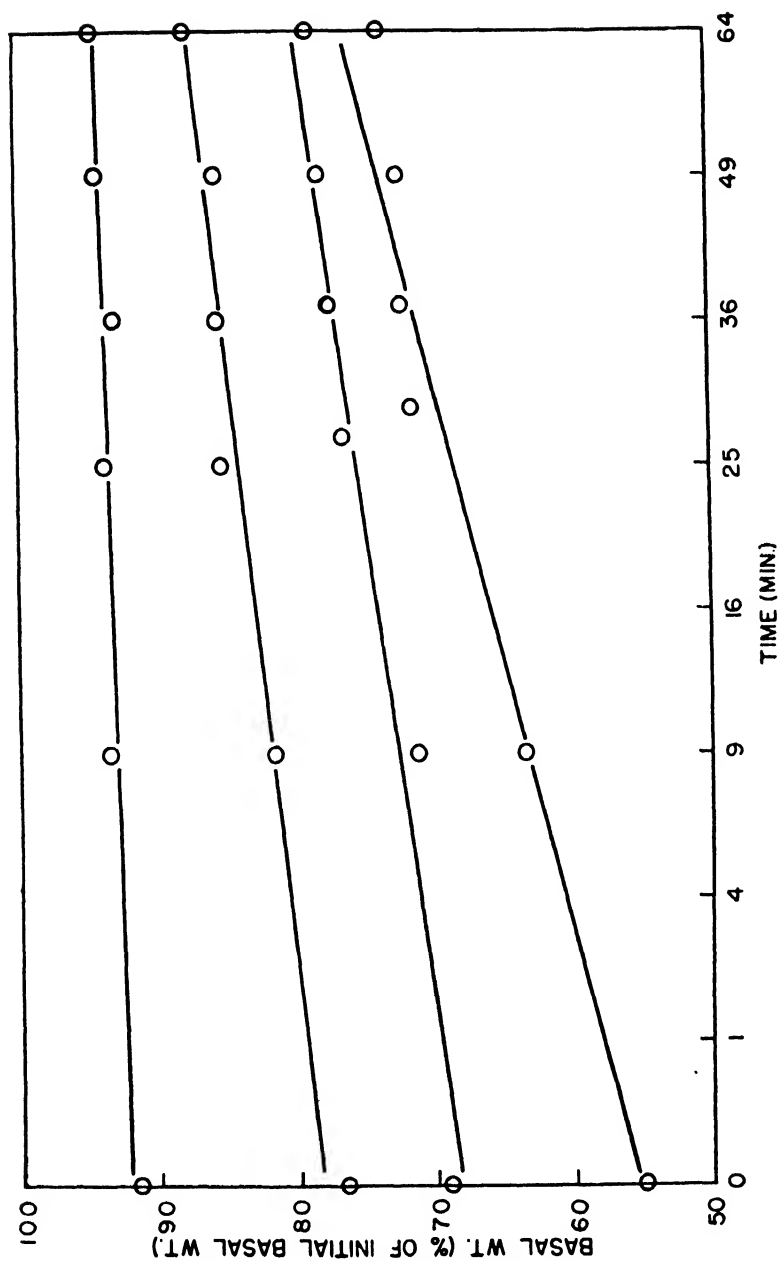


FIG. 7.—The weight of worms recovering from different levels of dehydration is proportional to \sqrt{t} . The points of each curve represent the mean weight of 3 worms.

The intake per minute at the ninth minute of the recovery period is, therefore,

$$\frac{dI}{dt} = \frac{k}{2\sqrt{t}} = \frac{I}{7 \times 2\sqrt{t}} = \frac{I}{7 \times 2\sqrt{9}} \quad (3)$$

or, per hour,

$$\frac{dI}{dt} = \frac{I \times 60}{7 \times 2 \times 3} = 1.43 I. \quad (4)$$

The slopes at 9 minutes were chosen simply as conveniently large ones for comparison. The initial slope (at $t = 0$), being infinity, attests to the inadequacy of equation

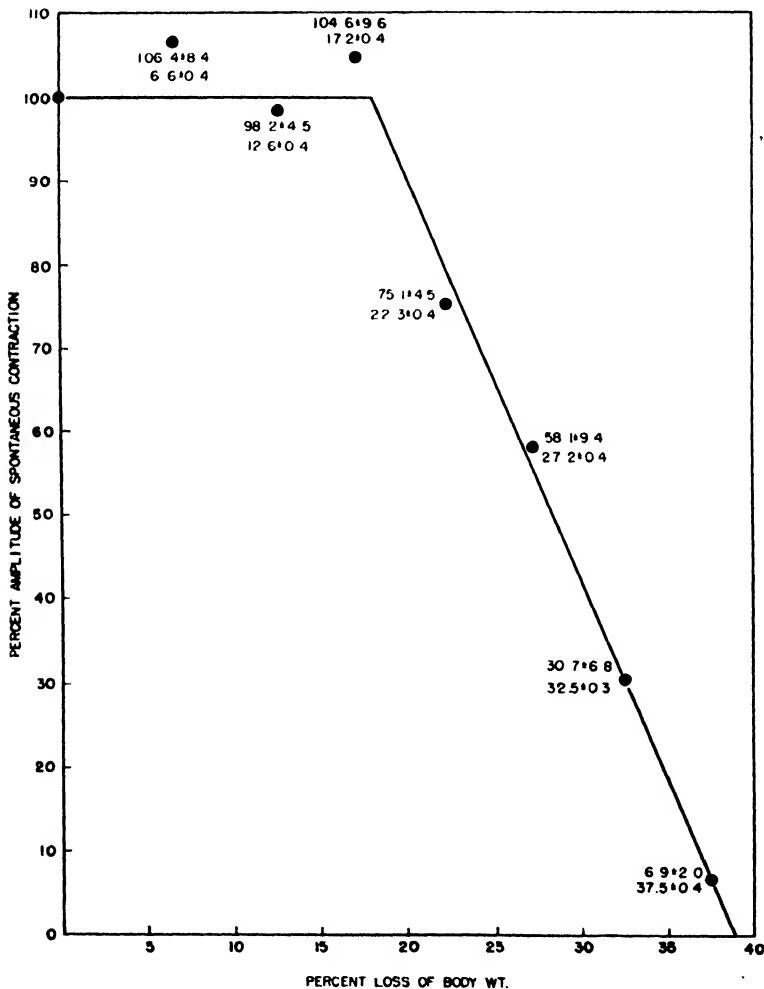


FIG. 8. - Relation between the amplitude of spontaneous contractions and the loss of body weight which obtained under the conditions stated in the text. Values of the ordinate and abscissa are given with their standard errors for 9 worms.

(1), and that slope obviously cannot be used. At zero dehydration under the conditions of these experiments the net intake of the integument is equal to the nephridial output (the worm is in water balance), i.e.,

$$I = ct, \quad (5)$$

where

$$c = 2.55 \text{ per cent per hour.}$$

Therefore,

$$\frac{dI}{dt} = c = 2.55 \text{ per cent of the basal body weight per hour at all times.} \quad (6)$$

Although a worm is reduced to basal weight, it is not dehydrated with respect to tissue water; so it is believed that basal weight represents the same water balance as gross weight. The integument seemed to vary its rate of intake only in response to dehydration of body tissue.

SPONTANEOUS CONTRACTIONS

It was thought interesting to study some activity in the earthworm which might be related to the state of tissue dehydration and hence to the rate at which the integument takes in water. The activity chosen was measured by recording on a kymograph the amplitude of the spontaneous longitudinal contractions of a worm's body when suspended by threads passed through one of the anterior and one of the posterior segments. The anterior end was suspended uppermost. Handling of the worm during threading tends to reduce the worm to basal weight, so that evaporation is largely loss of tissue water. The tension applied by the recording lever of the kymograph was approximately 0.3 gm. The amplitude of the contractions was measured by the distance between the highest and the lowest of the points obtained on the kymogram in a 2-minute run which was taken every 20 minutes, at which times the worm was weighed. Figure 8 indicates the relation between the amplitude of these contractions and the loss of body weight. There is no evidence that the same curve holds for worms recovering from water deficits.

DISCUSSION

The excretion rate of the intestine is obtained in terms of basal weight by multiplying the rates, which above were based on gross weight, by the factor 113.4/100.0 (13.4 per cent is the amount of fluid lost in reducing worms to basal weight). The value is 0.25 per cent of the basal body weight per hour. This amounts to only one-tenth of the rate for the sum of the nephridial units plus the rate of any gut excretion which may go on under these conditions, and it becomes apparent that the water exchanges of the two systems are of different orders of magnitude. The temperatures at which gut and nephridial determinations were made were not strictly alike. It is possible that the gut is not concerned primarily with maintenance of water balance but rather with the formation of digestive juices or the rendering of the feces fluid, in order that, as Darwin (1881) has indicated, the excrement may be spread about the burrow walls by the posterior end, which is used much as a trowel.

When a worm is reduced to basal weight, it merely suffers loss of a dead water load. Its removal reduces body volume. Since it is believed that the volume lost has come from

the lumina of the nephridia, we can, from the number of nephridia per specimen, estimate the mean capacity of the muscular duct plus any other part of the nephridium which may act as a storage place for dischargeable fluid (the ampulla may serve in some measure). The number of segments in 7 adult worms, ranging from 2.42 to 6.93 gm. basal weight, averaged 143 ± 4 . The number of nephridia per worm may be expressed as $N = 2s - 8$, where s represents the number of segments. N therefore averages 278 nephridia. Knowing the amount of fluid expelled when the average worm is reduced from gross to basal weight (13.4 per cent), we can determine the capacity of the storage space of an average nephridium as $13.4 \text{ per cent} \div 278$ or 0.048 per cent of the basal body weight, which in a 4-gm. worm constitutes a volume of 1.9 cmm. Gegenbaur (1853) gives plates and dimensions of *Lumbricus* nephridia in worms of unstated sizes which could accommodate this volume of fluid. Since the reservoir is effectively cylindrical, the volume varies as the square of the radius, so that with small changes of radius, comparatively large changes of content may be accommodated. A few observations by the writer corroborate the hypothesis that the nephridial reservoir is capable of holding the predicted water stores.

The average length of time required by a basal worm to attain gross weight is found from the figures for average loss from gross to basal weight and the nephridia excretion rate,

$$13.4 \text{ per cent} \div \frac{2.55 \text{ per cent}}{\text{hour}} = 5.25 \text{ hours.}$$

Possible factors affecting diurnal fluctuations of gross and basal weights may be summarized as (1) reservoirs varying in content as a result of periodic, partial expulsion of liquid; (2) loss of salts to distilled water, accompanied by loss of water, leading to a downward weight trend; (3) intestinal excretion, the effect of which may have been seen in the fluctuations of basal worms. The average excretion rate of the intestine (0.25 per cent per hour) could by accumulating in the gut give rise to approximately a 6 per cent weight increase in 24 hours, while the average change of basal weight seen in this time was only 2.3 per cent, an amount easily accounted for by intestinal fill; (4) small losses from the gut or body cavity in handling; (5) failure to attain the same degree of discharge from the nephridial battery in successive reductions to basal weight.

It was early thought that the nephridia served respiratory functions, Gegenbaur (1853) first suggesting their excretory role. But the nephridia may play an indirect part in respiration by keeping the integument moist. It may generally be observed that clusters of worms found in earth are quite moist. It has been held that exudation of coelomic fluid (and mucus glands) provides moisture, but it would seem more likely that coelomic-fluid exudation rather depends on irritating influences. If, during wet weather, earthworms store fluid in the nephridia, fractions of this might then be released at subsequent times. If the volume of fluid which may be contained in the gut is of the order of half of the gut volume (the remainder of the gut containing earth and food), the possible total store is 13 per cent + 8 per cent, or one-fifth of the body weight, or about one-quarter of the actual tissue water in the body. No data were collected to show the effect on the excretion rate or the storage space of the salts and other osmotic pressure-exerting substances which may exist in soils.

It is important to know the error which may be introduced by the use of gross, rather

than of basal, weights; but at the same time it should be stressed that the weight of a basal worm, too, is actually made up partly of an unknown dead water load in the gut which may be 15 per cent of the body weight.

The spontaneous contractions during dehydration represent part of a syndrome of dehydration described by Wolf (1938). The "rolling" phase in which elements of contracted longitudinal musculature travel in a circular sense about the body was here observed, and when a worm was lifted from its support and placed on a substratum, active rolling ensued.

SUMMARY

1. The mean diurnal fluctuations of gross and basal weights are 3.2 ± 0.3 and 2.3 ± 0.3 per cent of basal body weight, respectively. Both weights show a parallel downward trend in distilled water, over periods of 16 days, which may be due to permanent loss of salts and water during starvation.

2. The fraction (L_n) of fluid remaining in the nephridial reservoirs after n handlings was found to be given by $L_n = L_i (1-a)^n$, where L_i is the initial content and a had the value 0.631.

3. At 15° and 16° C. the gut was found to excrete fluid at approximately 0.25 per cent of a worm's basal body weight per hour, and this could be extruded through a mouth cannula until a maximum pressure of approximately 4.5 cm. of water was attained.

4. The gut was found not to contribute to any appreciable loss of weight, in worms undisturbed in water for some time, upon subsequent handling.

5. At 19° and 20° C. the nephridial battery of an earthworm was found to excrete fluid at a minimal rate of 2.55 ± 0.14 per cent of the basal body weight per hour, approximately ten times the rate of the gut.

6. The average difference between gross and basal weight amounted to 13.4 ± 0.9 per cent of the basal body weight. On this basis the storage capacity of an average nephridium may be estimated as 0.048 per cent of the basal body weight.

7. In worms in water the rate of net intake by the integument at given times increases with deficits of body water previously imposed by desiccation in air.

8. The amplitude of spontaneous contractions is unaffected by robbing a worm of as much as 18 per cent of its body weight of water, but it falls off in direct proportion thereafter, as the water loss progresses until no movement is evident when about 40 per cent of body weight has been lost.

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METHODS FOR EVALUATION OF HEAD TYPES IN PLANARIANS

(Twelve figures)

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Students of regeneration have for many years been confronted with the problem of assigning a quantitative measure to the anterior structures resulting from the regulation of experimentally isolated fragments of the body of triclad flatworms. In some of the American species the reaction appears to be of an "all-or-none" type (Buchanan, 1933, 1936, 1938) in that either normal heads are regenerated or none at all, while in planarians the regenerating fragments form a series of anterior structures graded from the normal head to a complete absence of any headlike organ or new tissue. Child (1911, 1912) distinguished five types of these anterior regenerated structures: normal; teratophthalmic, in which the head is of normal shape but the eyes are either decidedly asymmetrical in size or exhibit various degrees of fusion; teratomorphic, in which there is a single median eye and in which the shape of the head varies from the normal with laterally or anterolaterally placed auricles to a pointed anterior outgrowth; anophthalmic, without eyes but possessing a pointed outgrowth of new tissue; and acephalic or headless, in which there is no outgrowth of new tissue, the anterior end being rounded and the wound contracted and filled with scar tissue. These types of regenerates have been illustrated many times in the publications of Child and of his students.

It has been adequately demonstrated by a number of workers (Buchanan, 1922; Hinrichs, 1924; Rulon, 1936, 1937, 1938; Miller, 1937) that these types of anterior outgrowths form a more or less continuous series, for agents or conditions which favor the formation of heads shift the observed experimental outgrowths toward the upper (normal) end of the scale, while agents or conditions which inhibit head formation shift the observed types toward the lower (acephalic) end of the scale.

The concept of "head frequency" as an expression of the result of regeneration was given a roughly quantitative measure when Child and his students assigned to the head types arbitrary values. Various scales have been used: 100, 80, 60, 40, 20; 50, 40, 30, 20, 10; 5, 4, 3, 2, 1; etc., differing only in the choice of scale units, all of them identical in that in each one the successive steps in inhibition of a head are separated by equal intervals along an arithmetic scale. The use of these scales has made possible the derivation of a single, numerical expression for the result of an experiment involving numbers of pieces by simply taking as the "head-frequency index" the numerical average of the arbitrary values assigned to the types of heads regenerated. Thus, in using a scale of 50, 40, 30, 20, 10, a head-frequency index of 50 would indicate that all pieces had regenerated normal heads, one of 10 that all pieces were acephalic, and one of 35 that the "average" head resulting was intermediate between the teratomorphic and teratophthalmic types.

Valuable as this scheme has been in making possible a roughly quantitative estimate of the result of anterior regeneration of planarian fragments, it remains in some degree unsatisfactory as long as the numerical values assigned are arbitrary. The assigning of

numerical values with equal intervals between the values for successive categories implies that each head type differs from the one above or below it in the scale in some mathematical fashion. If this is true, the relationship may be interpreted theoretically by some such graphical situation as that suggested in Figure 1. The relationship here illustrated is such that equal advances in conditions favoring head formation elicit responses in anterior outgrowths represented by equal successive steps in the perfection of heads.

If the above hypothesis is true, then under conditions that permit the formation of all types of heads the intermediate categories will be well represented. That such is not the case is suggested by the observations of different workers. Results published by

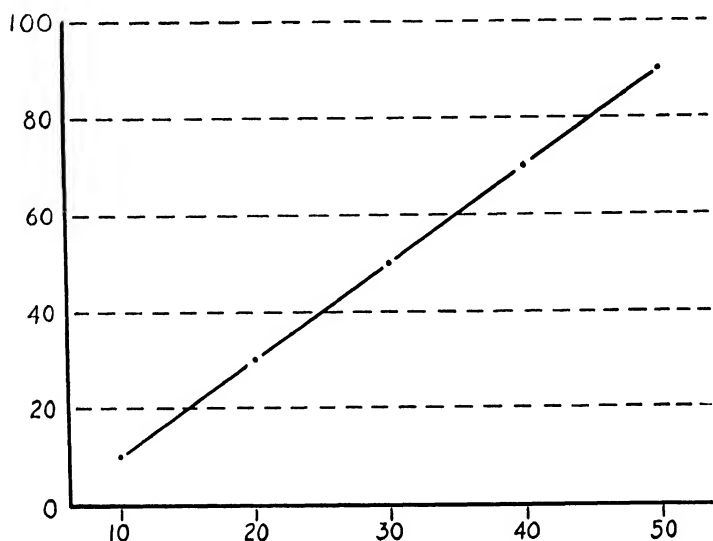


FIG. 1. Diagram of the hypothetical relationship between head-frequency indices (abscissas) of the equal-interval scale and the ranges of the head types (ordinates), based on the assumption that the ranges for the different head types are equal. The ranges are shown on a scale of 0 to 100; 0-20, acephalic; 20-40, anophthalmic; 40-60, teratomorphic; 60-80, teratophthalmic; 80-100, normal.

Sivickis (1923) and Buchanan (1938) are characteristic of the experience of others. In the tabulations of the types of heads observed by these two workers it is clear that anophthalmic outgrowths occur in fewer numbers than do normals, teratophthalmics, or acephalics and that teratomorphic heads are observed relatively infrequently.

It is obvious that to be of most use in expressing head frequency accurately the numerical scale adopted must bear some relation to the size of the groups represented. The use of an equal-interval scale would be justified if the same change in experimental condition that shifts normal heads to teratophthalmic also shifted teratophthalmic to teratomorphic and so on down the scale. If this relationship as revealed by the observed frequency of occurrence of heads of different types does not exist, then a revision of the indices should be made, and the types of heads occurring in fewer than the expected numbers should be allotted narrower "bands" in the entire scale. The numerical steps between successive grades in head perfection (or inhibition) will then be unequal.

The writer, in being confronted with the evaluation of the results of regeneration of large numbers of fragments of *Dugesia dorocephala* (= *Euplanaria dorocephala*) and *D. tigrina* (= *E. maculata*),¹ has been impressed not only by the inequality in numbers of individuals occurring in the different recognizable categories but also by the inadequacy of the scale of arbitrarily assigned values for discriminating between experiments in which the differences in head frequency are slight or not obvious. It is this experience that has led to the present attempt to develop more satisfactory methods for the evaluation of head-frequency data.

EXPERIMENTAL OBSERVATIONS

As a consequence of a series of regeneration experiments undertaken on *D. dorocephala* and *D. tigrina*, records of anterior structures formed by considerable numbers of fragments of members of these two species have been made available. Fragments used

TABLE 1
RECORD OF AN EXPERIMENT WITH *D. dorocephala* TO SHOW DISTRIBUTION OF
REGENERATED STRUCTURES TYPICALLY OBSERVED

(Size of pieces: one-eighth of 12-13-mm. worms after removal of heads and tails)

Level	Number of Pieces	Normal	Teratophthalmic	Teratomorphic	Anophthalmic	Acephalic	Dead
A.....	10	8	2
B.....	10	2	4	2	2
C.....	10	1	1	4	4
D.....	10	1	2	7
E.....	10	6	1	3
F.....	10	1	1	1	3	4
G.....	10	1	4	1	2	2
H.....	10	6	4

were one-eighth of the length of the body after removal of the head and tail; specimens of *D. dorocephala* used were approximately 12-13 mm. in length, and of *D. tigrina* approximately 10-11 mm. Fragments were considered in units of 10 from each of the eight levels of the body; the 80 fragments which resulted from cutting up 10 worms constituted an experiment. Records were kept on this basis. Data are here considered from 60 experiments with *D. dorocephala* (a total of about 4,800 fragments) and about 150 experiments with *D. tigrina* (a total of about 12,000 fragments).

A representative record of a single experiment with *D. dorocephala* is shown in Table 1. It is to be observed that at levels of the body showing high head frequency (levels A and H) the resulting anterior outgrowths as determined after 10 days are all of the higher orders of heads, usually distributed between normals and teratophthalmics. At levels showing lowest head frequency (level D) acephalic forms predominate. Occasionally, however, there are to be found units of 10 pieces from the same level of the body and isolated under as nearly as possible the same experimental conditions in which both extremes of the head-type scale are represented, together with a scattering of heads of intermediate type (levels B, F, and G). The conditions under which these

¹ See Hyman (1939) for the taxonomic history of these two species.

fragments have regenerated have been such that they have permitted the formation of all types of outgrowths which Child has grouped into his five categories.

From the records of the experiments all units of 10 pieces which have shown the two extremes of the range from acephalic to normal have been selected for use in the calculations to be discussed under Method I, without regard to the fact that in many of these units one or more of the intermediate head types may not be represented (Table 1, level B, for example). These have been totaled separately for *D. dorotocephala* and *D. tigrina*, with the result tabulated in Table 2. For *D. dorotocephala* a total of

TABLE 2
DISTRIBUTION OF HEAD TYPES IN EXPERIMENTAL UNITS
SHOWING RANGE FROM NORMAL TO ACEPHALIC

HEAD TYPE	<i>D. dorotocephala</i>		<i>D. tigrina</i>	
	Number	Per Cent	Number	Per Cent
Acephalic.....	424	35.01	551	20.18
Anophthalmic.....	99	8.18	446	16.34
Teratomorphic.....	72	5.95	153	5.60
Teratophthalmic....	356	29.40	613	22.45
Normal.....	260	21.47	967	35.42
Total.....	1,211	100.01	2,730	99.99

1,211 regenerated fragments are included in this tabulation. Of these 424, or 35.01 per cent, are acephalic; 99, or 8.18 per cent, are anophthalmic; 72, or 5.95 per cent, are teratomorphic; 356, or 29.40 per cent, are teratophthalmic; and 260, or 21.47 per cent, are normal heads. In the tabulation for *D. tigrina* 2,730 fragments are included, with the distribution of regenerated structures as shown in Table 2. It is obvious from these data as from the records of Sivickis (1923) and Buchanan (1938) that the anophthalmic and teratomorphic heads occur with much lower frequency than do the other types.

NEW METHODS FOR EXPRESSION OF HEAD FREQUENCY

METHOD I

The method here presented is a means for assigning definite values to heads of different types. These indices differ from those of the older scale in that they are assigned on the basis of the observed frequency of occurrence of the head types in experimental units showing the whole range of the scale. The indices are not separated by equal intervals along the scale but by distances determined by the widths of the ranges of the different categories.

In pieces isolated from the same level of different worms from a homogeneous stock individual and uncontrollable differences in physiologic condition of the worms, in the size of the piece, or in the conditions in the immediate vicinity of the regenerating fragments or other factors appear to be sufficient to account for the variation in the types of heads regenerated. If each factor acting has the same effect at all levels of the scale, then the frequency distribution of regenerated structures should be that of a normal probability curve, the acephalic and normal forms occupying opposite extremes of the

curve and the intermediate classes of heads lying between. It is further assumed that the thresholds between successive grades of head development are at relatively fixed points on the scale. Such a distribution of the data for *D. dorocephala* found in Table 2 is presented in Figure 2.

From the available information on the percentages of the total population found in the respective classes it is possible to calculate, in terms of the standard deviation or in terms of an arbitrary scale of 0-100 units assigned to the total range, the limits (thresholds) and class ranges of the five separate classes of heads. For these calculations the

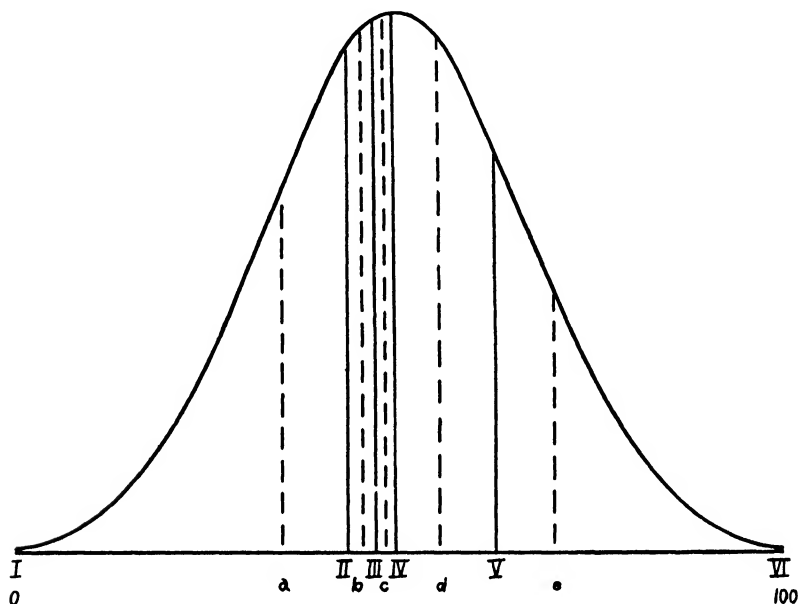


FIG. 2.—Observed frequency distribution of head types in *D. dorocephala* fitted to a normal probability curve. The distribution is based on a compilation of results from units of 10 pieces which show the whole range of head types, selected from the author's experiments. Solid lines represent the limits or thresholds between successive classes of head types; broken lines represent the class medians. Values for the abscissas of the class limits (I, II, . . . , VI) are given in Table 3 in terms of standard deviations and in terms of the scale of 0-100 assigned to the entire range. Values for the abscissas of the class medians (a, b, . . . , e) are given in Table 4.

normal-curve area table in Chaddock (1925, p. 463) has been used. The values given in this table are such that 3.1 standard deviations from the mean include 49.93 per cent of the cases. This distance, measured both plus and minus from the mean, will therefore include 99.86 per cent or practically all the cases. This total range of 6.2 standard deviations is taken as equal to the 100 units of the arbitrary scale in which the final values are to be expressed.

The calculations for the procedure outlined below are set forth in Table 3. In column 2 are recorded the percentages of the total population present in each class. From these values the running sums (Σp) shown in column 3 are derived. Then 50 is subtracted from each of these values ($\Sigma p - 50$) to obtain the percentages between the mean and

the given threshold. These values are shown in column 4, the sign indicating on which side of the median each threshold lies. The abscissa of the unit normal probability curve, or inverse probability function (prf^{-1}), for each of these thresholds is determined in terms of the standard deviation (col. 5) by reference to a normal probability-curve area table such as that cited in Chaddock. The algebraic differences between the successive values in column 5 are recorded in column 6. These differences represent in terms of standard deviations the ranges of the classes. In column 7 these ranges are converted into the units of the arbitrary scale of 0-100 by multiplying them by $100/6.2$. Finally, by finding in column 8 the running sums of the values in column 7, the class limits or

TABLE 3
CALCULATION OF CLASS LIMITS AND RANGES
(*D. dorocephala*)

(1) Class	(2) Percentage in Each Class p	(3) Running Sum of (2) Σp	(4) Distance from Mean $\Sigma p - 50$	(5) Inverse Probability Function $prf^{-1}(4)$	(6) Class Range in σ	(7) Class Range in Scale Units $(6) \frac{100}{6.2}$	(8) Class Limits from Running Sum of (7)
Acephalic	35.01	(0.00)	(-50.00)	$-\infty (-3.1)$			0.00 (I)
Anophthalmic	8.18	35.01	-14.99	-0.385	2.715	43.79	43.79 (II)
Teratomorphic	5.95	43.19	-6.81	-0.171	0.214	3.45	47.24 (III)
Teratophthalmic	20.40	49.14	-0.86	-0.021	0.150	2.42	49.66 (IV)
Normal	21.47	78.54	+28.54	+0.791	0.812	13.10	62.76 (V)
Total	100.01	100.01	+50.01	$+\infty (+3.1)$	2.309	37.24	100.00 (VI)
Total	100.01				6.200	100.00	

thresholds are determined. The Roman numerals (I, II, . . . , VI) accompanying these values refer to the positions of these limits as indicated in Figure 2.

While the determinations outlined above locate the limits of the five classes with respect to the total range of the population, the values are in some ways not suitable for use in the expression of head frequency. For this purpose a value is desired which for any one class is more representative of the entire class than is the value for either of the thresholds limiting the class. Although not as constant a value as those for the thresholds determined above, the value for the median of each class has been chosen for this purpose. The reasons for this choice will be indicated more fully in a later section of this paper. The abscissas for the medians in terms of the arbitrary scale are determined from the class-percentage frequencies by calculations indicated in Table 4. From the

class percentages the medians are located in the population by finding the percentages of cases lying below the successive medians (col. 3). Then 50 is subtracted from each

TABLE 4
CALCULATION OF ABSCISSAS OF CLASS MEDIANS
(*D. dorotocephala*)

(1) Class	(2) Percentage in Each Class p	(3) Percentage below Successive Medians	(4) Distance from Median (3) - 50	(5) Inverse Probability Function $\Phi^{-1}(4)$	(6) Abscissa in σ (5) + 3.1	(7) Abscissa in Scale Units (6) $\frac{100}{6.2}$
Acephalic.....	35.01	17.55	-32.45	-0.933	2.167	34.95(a)
Anophthalmic.....	8.18	39.10	-10.90	-0.277	2.823	45.53(b)
Teratomorphic.....	5.95	46.17	-3.83	-0.096	3.004	48.45(c)
Teratophthalmic.....	29.40	63.84	+13.84	+0.354	3.454	55.71(d)
Normal.....	21.47	89.24	+39.24	+1.240	4.340	70.00(e)

of these values to locate them with respect to the median for the entire population (col. 4). By reference to the normal-curve area table (Chaddock, 1925) the inverse prob-

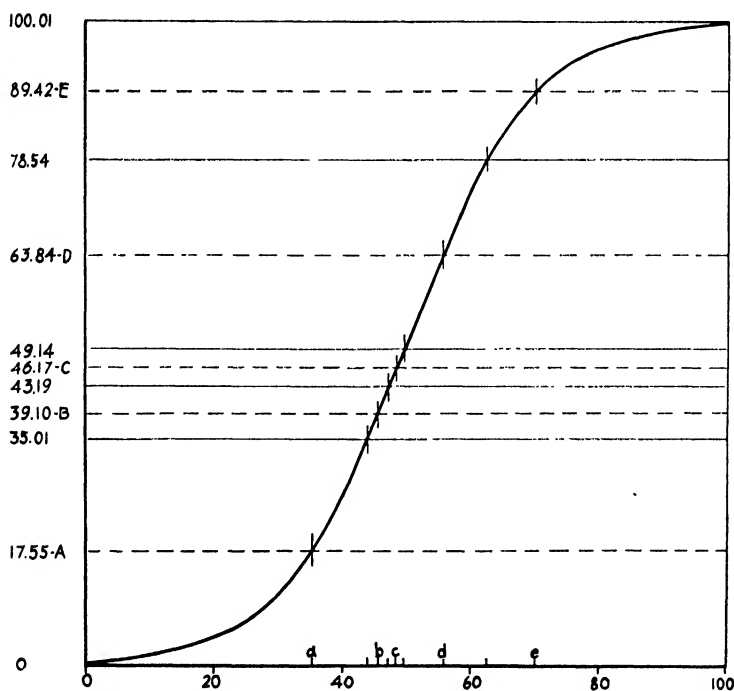


FIG. 3.—Relationship between observed percentage distribution of head types (ordinates) and values determined by Method I for the class limits and medians (abscissas). Values for the abscissas of the class limits (I, II, . . . , VI) are given in Table 3 and for the medians (a, b, . . . , e) in Table 4.

ability function of each of these points is found (col. 5), thus expressing the locations in terms of standard deviations from the mean. The addition of 3.1 to these values (col. 6) locates the individual class medians with respect to the extreme lower end of the scale, and these values are converted to the arbitrary-scale units by multiplying them by $100/6.2$ (col. 7). The key letters (*a*, *b*, . . . , *e*) accompanying the values in this column refer to the locations indicated in Figure 2.

Using the values determined above for the abscissas of the class limits and medians, a curve may be constructed to show their relationship to the percentage distribution of head types. This curve is shown in Figure 3 and is to be contrasted to the hypothetical

TABLE 5
CALCULATION OF CLASS LIMITS AND RANGES
(*D. tigrina*)

(1) Class	(2) Percentage in Each Class p	(3) Running Sum of (2) Σp	(4) Distance from Mean $\Sigma p - 50$	(5) Inverse Probability Function $prf^{-1}(4)$	(6) Class Range in σ Δ	(7) Class Range in Scale Units $(6) \frac{100}{6.2}$	(8) Class Limits from Running Sum of (7)
Acephalic . . .	20 18	(0 00)	(-50 00)	$-\infty (-3.1)$			0 00
Anophthalmic . . .	16 34	20 18	-29 82	-0 835	2.265	36 53	36 53
Teratomorphic . .	5 60	36 52	-13 48	-0.345	0 400	7 00	44 43
Teratophthalmic	22 45	42 12	-7 88	-0 199	0 146	2 35	46 78
Normal	35 42	64 57	+14.57	+0 374	0 573	9 24	56 02
Total . . .	99 99	99 99	+49 99	$+\infty (+3.1)$	2.726	43 97	99 99
	99 99				6 200	99 99	

situation suggested in Figure 1. Since in Figure 1 the intervals between successive values assigned to the head types are equal and the distances between the thresholds are represented as of equal size, the curve expressing the relationship between the two is a straight line. In Figure 3 the bands for inclusion of the classes correspond in width to the experimentally observed frequency of occurrence of the head types, the values for the medians and for the thresholds are as determined above, and the curve of relationship is of the sigmoid type.

From the data recorded in Table 2 for *D. tigrina* a series of values for the class ranges, limits, and the medians has been determined for this species. The method of procedure is precisely the same as has been outlined above for *D. dorotocephala*. The calculations and final values are shown in Tables 5 and 6. Since the percentage distribution of the

head types differs from that for *D. dorocephala*, the values are not the same as for *D. dorocephala*—a result to be anticipated. If the values determined for *D. tigrina* are plotted against the class frequencies, the points will fall upon the same curve as

TABLE 6
CALCULATION OF ABSISSAS OF CLASS MEDIANS
(*D. tigrina*)

(1) Class	(2) Percentage in Each Class p	(3) Percentage below Successive Medians	(4) Distance from Median (3) - 50	(5) Inverse Probability Function $\text{prf}^{-1}(4)$	(6) Abscissa in σ (5) + 3.1	(7) Abscissa in Scale Units (6) $\frac{100}{6.2}$
Acephalic	20.18	10.09	-39.91	-1.276	1.824	29.42
Anophthalmic	16.34	28.35	-21.65	-0.572	2.528	40.78
Teratomorphic	5.60	39.32	-10.68	-0.271	2.829	45.63
Teratophthalmic	22.45	53.55	+3.55	+0.089	3.189	51.43
Normal	35.42	82.28	+32.28	+0.926	4.026	64.92

that shown for *D. dorocephala* in Figure 3. This must of necessity be so, since both series of values are determined in the same fashion from constants for a normal probability curve and since the curve of relationship has a definite mathematical relationship to the normal curve.

METHOD II

The indices developed in the preceding section depend for their determination upon the availability of a body of data from experiments in which the individual groups of pieces are small. Wright (private communication) has applied to the problem an elaboration of the method he has used for the determination of values for the thresholds between different degrees of polydactyly in guinea pigs (Wright, 1934). His method makes possible the determination of values for the ranges and limits of the three intermediate classes of heads from data on experiments with large numbers of pieces from a homogeneous stock. Having made this determination, it is then possible to describe the population of regenerated structures by locating its mean relative to the total range of the three classes and by determining its variability by finding the standard deviation from this mean.

The assumptions underlying the method are the same as those underlying Method I, namely, (1) that the frequency distribution of head types should be that of a normal probability curve relative to the scale of factor combinations determining head types and (2) that the thresholds between successive grades of head development are at fixed points on the scale.

Wright applied the method to data on *D. dorocephala* from the observations of several students. From the three different bodies of data the values determined for the class ranges were averaged after proper weighting to take into account the reliability of the different determinations. The final estimate was that the ranges of the three classes considered (anophthalmia, teratomorphia, and teratophthalmia) were in the proportion of 0.36:0.14:0.50, expressed as fractions of the total range of the three classes.

It is of interest to apply the method suggested by Wright to the whole of the mass of data from which a selection was made for use in the determinations of Method I. The writer wishes at this point to acknowledge his indebtedness to Dr. Sewall Wright for his painstaking care in explaining the method and for his permission to make use of the unpublished work.

In Table 7 are summarized the results from the 60 separate experiments on *D. dorotocephala* previously cited. Each experiment consisted of an original 80 pieces from 10 worms, 10 pieces from each of the 8 levels of the body indicated. The numerical and

TABLE 7
DISTRIBUTION OF HEAD TYPES: TOTALS FOR PIECES
SURVIVING FROM SIXTY EXPERIMENTS
(*D. dorotocephala*)

Level	Normal	Teratophthalmic	Teratomorphic	Anophthalmic	Acephalic	Total
A {No.	280	240	12	2	10	544
{Per cent. . . .	51.47	44.12	2.21	0.37	1.84	100.01
B {No.	60	243	36	51	183	573
{Per cent. . . .	10.47	42.41	6.28	8.90	31.94	100.00
C {No.	7	52	34	94	376	563
{Per cent. . . .	1.24	9.24	6.04	16.69	66.78	99.99
D {No.	9	23	23	40	469	564
{Per cent. . . .	1.60	4.08	4.08	7.09	83.16	100.01
E {No.	15	81	47	71	358	572
{Per cent. . . .	2.62	14.16	8.22	12.41	62.59	100.00
F {No.	10	85	41	77	367	580
{Per cent. . . .	1.72	14.66	7.07	13.28	63.28	100.01
G {No.	51	162	45	81	258	597
{Per cent. . . .	8.54	27.14	7.54	13.57	43.22	100.01
H {No.	281	232	10	13	25	561
{Per cent. . . .	50.09	41.35	1.78	2.32	4.46	100.00

percentage distribution of head types formed by the survivors from the original 600 pieces at each of the 8 levels are shown.

From the data presented in Table 7 estimates are made of the ranges of the five categories for each of the eight levels. For the sake of brevity in the tabulation and in the discussion to follow, these categories have been designated as *A*, acephalic; *B*, anophthalmic; *C*, teratomorphic; *D*, teratophthalmic; and *E*, normal. From the running sum of the percentages in each class 50 is subtracted to obtain the percentage between the mean and the given threshold. The abscissas of the unit normal probability curve (inverse probability function) corresponding to each of these frequencies is determined from a normal-curve area table (Chaddock, 1925). The difference between successive

values, taking account of signs, is the required class range in terms of the standard deviation. This procedure is the same as that used in Method I. The calculations are indicated in Table 8.

TABLE 8
ESTIMATION OF CLASS RANGES
(*D. dorotocephala*)

Level of Body	Class	Percentage in Class p	Summation of Percentages Σp	Distance from Median $\Sigma p - 50$	Inverse Probability Function $prf^{-1}(\Sigma p - 50)$	Range
A.....	A	1.84	1.84	-48.16	-2.088	1.012
	B	0.37	2.21	-47.79	-2.012	0.076
	C	2.21	4.42	-45.58	-1.704	0.308
	D	44.12	48.54	-1.46	-0.036	1.068
	E	51.47	100.01	+50.01	+3.1	3.136
B.....	A	31.94	31.94	-18.06	-0.469	2.631
	B	8.90	40.84	-9.16	-0.232	0.237
	C	6.28	47.12	-2.88	-0.073	0.159
	D	42.41	89.53	+39.53	+1.255	1.328
	E	10.47	100.00	+50.00	+3.1	1.845
C.....	A	66.78	66.78	+16.78	+0.434	3.534
	B	16.60	83.47	+33.47	+0.974	0.540
	C	6.04	89.51	+39.51	+1.254	0.280
	D	9.24	98.75	+48.75	+2.240	0.986
	E	1.24	99.99	+49.99	+3.1	0.860
D.....	A	83.16	83.16	+33.16	+0.960	4.060
	B	7.00	90.25	+40.25	+1.296	0.336
	C	4.08	94.33	+44.33	+1.583	0.287
	D	4.08	98.41	+48.41	+2.158	0.575
	E	1.60	100.01	+50.01	+3.1	0.942
E.....	A	62.59	62.59	+12.59	+0.321	3.421
	B	12.41	75.00	+25.00	+0.675	0.354
	C	8.22	83.22	+33.22	+0.963	0.288
	D	14.16	97.38	+47.38	+1.940	0.977
	E	2.62	100.00	+50.00	+3.1	1.160
F.....	A	63.28	63.28	+13.28	+0.339	3.439
	B	13.28	76.56	+26.56	+0.725	0.386
	C	7.07	83.63	+33.63	+0.979	0.254
	D	14.06	97.69	+47.69	+2.118	1.139
	E	1.72	100.01	+50.01	+3.1	0.982
G.....	A	43.22	43.22	-6.78	-0.171	2.929
	B	13.57	56.79	+6.79	+0.171	0.342
	C	7.54	64.33	+14.33	+0.367	0.196
	D	27.14	91.47	+41.47	+1.370	1.003
	E	8.54	100.01	+50.01	+3.1	1.730
H.....	A	4.46	4.46	-45.54	-1.700	1.400
	B	2.32	6.78	-43.22	-1.492	0.208
	C	1.78	8.56	-41.44	-1.364	0.128
	D	41.35	49.91	-0.09	-0.002	1.362
	E	50.09	100.00	+50.00	+3.1	3.102

From the determinations made above it is clear that the eight populations taken from eight different levels of the body differ from each other. For the final estimate desired of the ranges of the three intermediate classes the ranges are weighted. For weighting, the percentage above or below the class in question, whichever is smaller, is multiplied by the value for the range determined in Table 8. A more accurate weighting could be obtained by multiplying also by the total number in each population, but since the populations are roughly of the same size this step is not included. The weighting and final weighted averages are shown in Table 9. The final estimate shows that the ranges of classes *B*, *C*, and *D* are in the proportion of 0.19:0.12:0.69 when expressed as frac-

TABLE 9
ESTIMATE OF RANGES OF CLASSES *B*, *C*, AND *D*; WEIGHTING OF
ESTIMATES FOR THE EIGHT LEVELS

(*D. dorsotocephala*)

LEVEL	<i>B</i>		<i>C</i>		<i>D</i>	
	Weight	Range	Weight	Range	Weight	Range
A	1 84	0 076	2 21	0 308	4 42	1 668
B	31 94	0 237	40 84	0 159	10 47	1 328
C	16 52	0 540	10 48	0 280	1 24	0 986
D	9 76	0 336	5 68	0 287	1 60	0 575
E	24 00	0 354	16 78	0 288	2 62	0 977
F	23 45	0 386	16 38	0 254	1 72	1 139
G	43 22	0 342	35 68	0 196	8 54	1 003
H	4 46	0 208	6 78	0 128	8 56	1 362
Total	155 19	134 83	39 17
Weighted averages	0 3426	0 2121	1 2295

Total range of classes *B*, *C*, and *D* = 1.7842

Proportions of total range occupied by classes

B, *C*, and *D* = 0.19:0.12:0.69

tions of the total range of the three categories. It is of interest at this point to indicate the close agreement of this proportion to that of 0.18:0.13:0.69 arrived at from the estimate of ranges made by Method I.

Having arrived at an evaluation of the ranges of classes *B*, *C*, and *D*, it is now possible to locate the distribution of any of the eight populations relative to them. Each population is divided into three classes in such a fashion that the numbers in the classes are as nearly equal as possible. For the middle class the inverse probability function ($prf^{-1}[\Sigma p - 50]$) is found for the upper and lower limits (values already recorded in Table 8). The reciprocal of the difference between these values, multiplied by the theoretical class range, is the standard deviation. The mean of the population relative to the class limits is located by multiplying either the upper or the lower class limit, as ob-

tained above, by the standard deviation. For all possible middle classes used in making the application the following are the theoretical class limits:

Middle Class	Theoretical Range
<i>B</i>	0.19
<i>C</i>	0.12
<i>B</i> + <i>C</i>	0.31
<i>D</i>	0.60
<i>C</i> + <i>D</i>	0.81
<i>B</i> + <i>C</i> + <i>D</i>	1.00

The actual application of the method to the determination of the population means and standard deviations is shown in Table 10, A. Figures are here given for the eight levels studied as well as for the selected group studied in Method I.

A summary of the values for the means and standard deviations of the nine populations is shown in Table 10, B. The means are located from the values for the upper-class limits on a scale such that the lower threshold for class *B* (anophthalmia) is at 0 and the upper limit for class *D* (teratophthalmia) is at +1.

Method II provides a way for describing the variability of populations made up of assorted head types and for locating the means of the populations relative to the ranges of the three intermediate classes. Having described two populations (experimental and control groups, for instance) in this fashion, it is then possible to compare them. Examples will be given in the following section on the application of the method.

APPLICATION OF METHODS TO THE EVALUATION OF HEAD-FREQUENCY DATA

In the following paragraphs and accompanying graphs and tables are presented examples of the application of different methods to the evaluation of existing head-frequency data. All the data used are taken from work on *D. dorotocephala*. For calculations on the basis of the older scale of equal intervals, values of 50, 40, 30, 20, 10 have been assigned to the head types from normal to acephalic. For calculations on the basis of values determined by Method I of this paper the values given in Table 4 have been rounded off to the nearest whole number so that the indices used have been as follows: normal, 70; teratophthalmic, 56; teratomorphic, 48; anophthalmic, 46; and acephalic, 35. It is assumed for the sake of illustration that the ranges of the different classes for the stocks used by other workers are the same as those of the stock used by the author. As will be pointed out later, there are sufficient reasons for believing that this assumption is not always a valid one, a fact to be borne in mind in considering the comparisons here made. In the sets of graphs intended for comparison the range 10-50 in the one case and 35-70 in the other have been allotted the same distance along the ordinate in order that the graphs may be directly compared.

Figures 4 and 5 are graphical presentations of data recorded by Child (1916, Table 2, p. 106). The ordinates in each figure represent head-frequency indices calculated for pieces from the body-levels indicated as abscissas; data for Figure 4 have been calculated on the older scale of values, and for Figure 5 on the values determined by Method I. Very little difference in the curves appears as a consequence of the change in the indices. The new scale does, however, accomplish for most levels of the body a greater separation of the ordinates for experiment (broken lines) and control (solid lines). This separation

brings out more clearly the effect of the experimental treatment—in this case the subjection of the experimental pieces to cyanide.

TABLE 10
LOCATION OF THE DISTRIBUTIONS OF SEPARATE POPULATIONS
(*D. dorocephala*)

A. CALCULATION OF STANDARD DEVIATIONS AND LOCATION OF
MEANS RELATIVE TO CLASS LIMITS

<i>A pieces:</i>			<i>B pieces:</i>			<i>C pieces:</i>		
	Limits			Limits			Limits	
<i>C</i>	-1 704	-0 70	<i>C</i>	-0 073	-0 04	<i>A</i>	+0.434	+0.15
<i>D</i>	-0 036	-0 01	<i>D</i>	+1 255	+0 05	<i>B</i>	+0 974	+0.34
<i>D</i>	1 668	0 69	<i>D</i>	1.328	0 69	<i>B</i>	0 540	0 19
$\sigma = \frac{0.69}{1.668} = 0.41$			$\sigma = \frac{0.69}{1.328} = 0.52$			$\sigma = \frac{0.19}{0.54} = 0.35$		
<i>D pieces:</i>			<i>E pieces:</i>			<i>F pieces:</i>		
	Limits			Limits			Limits	
<i>A</i>	+0 060	+0 55	<i>A</i>	+0 321	+0 15	<i>A</i>	+0 339	+0 16
<i>B</i>	+1 296	+0 74	<i>C</i>	+0 963	+0 46	<i>C</i>	+0 979	+0 47
<i>B</i>	0 336	0 19	<i>B+C</i>	0.642	0 31	<i>B+C</i>	0.640	0.31
$\sigma = \frac{0.19}{0.336} = 0.57$			$\sigma = \frac{0.31}{0.642} = 0.48$			$\sigma = \frac{0.31}{0.64} = 0.48$		
<i>G pieces:</i>			<i>H pieces:</i>			<i>Method I group:</i>		
	Limits			Limits			Limits	
<i>A</i>	-0 171	-0 10	<i>C</i>	-1 364	-0 696	<i>A</i>	-0 385	-0 33
<i>C</i>	+0 367	+0 21	<i>D</i>	-0 002	-0 001	<i>D</i>	+0 791	+0 67
<i>B+C</i>	0 538	0 31	<i>D</i>	1.362	0 69	<i>B+C+D</i>	1 176	1 00
$\sigma = \frac{0.31}{0.538} = 0.58$			$\sigma = \frac{0.69}{1.362} = 0.51$			$\sigma = \frac{1.00}{1.176} = 0.85$		

B. SUMMARY AND LOCATION OF MEANS RELATIVE TO A SCALE ON
WHICH THE LOWER LIMIT FOR CLASS *B* IS AT 0, AND THE
UPPER LIMIT FOR CLASS *D* AT +1

Group	Mean	Standard Deviation	Group	Mean	Standard Deviation
<i>A pieces.</i>	+1 01	0 41	<i>F pieces.</i>	-0 16	0 48
<i>B pieces.</i>	+0 34	0 52	<i>G pieces.</i>	+0 10	0 58
<i>C pieces.</i>	-0 15	0 35	<i>H pieces.</i>	+1 00	0 51
<i>D pieces.</i>	-0 55	0 57	<i>Method I.</i>	+0 327	0 85
<i>E pieces.</i>	-0 15	0 48			

Data for the graphs shown in Figures 6 and 7 have been taken from Buchanan (1923, Table 2, p. 407). Ordinates for Figure 6 have been calculated by the older scale of indices and for Figure 7 by the indices of Method I. The curves for experimental (broken line)

and control pieces (solid line) show little change in their general form or in their position relative to each other as a result of the change in indices.

Data for the graphs shown in Figures 8 and 9 are taken from Hinrichs (1924, Table 5, p. 283). Figure 8 is on the basis of the older head-frequency indices, and Figure 9 on the basis of those of Method I. Attention here is directed to the effect of the experimental treatment (caffeine) on the head frequency of pieces at the B level. From Figure 8 it appears that the head frequency of pieces in the experimental group (broken line) is higher than that of the controls (solid line), but in Figure 9 the relationship is reversed.

TABLE 11
EVALUATION OF DATA FROM HINRICHS' EXPERIMENT
(*D. dorotocephala*)

A. COMPARISON OF MEANS DETERMINED BY USE OF HEAD-FREQUENCY INDICES

	A			B			C		
	Mean	$m_e - m_c$	P^*	Mean	$m_e - m_c$	P	Mean	$m_e - m_c$	P
Indices of equal-interval scale:									
Experimental.....	45.60	+1.6	0.0455	26.40	+0.80	0.500+	10.20	-2.20	0.230
Control.....	45.00			25.60			21.40		
Indices of Method I:									
Experimental.....	64.20	+1.08	0.2713	46.50	-0.32	0.500+	41.60	-1.95	0.109
Control.....	63.12			46.82			43.64		

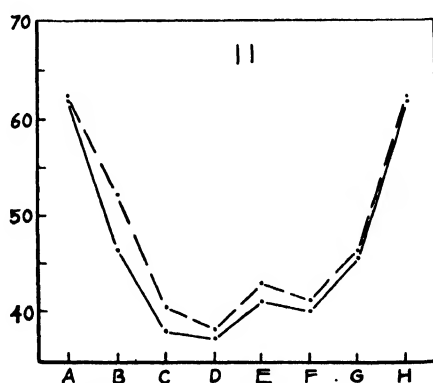
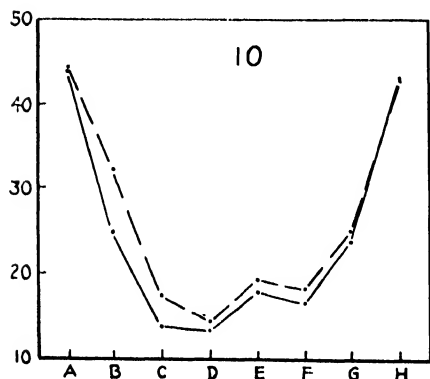
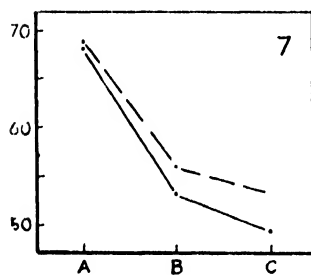
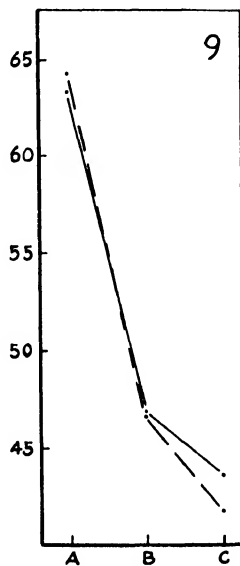
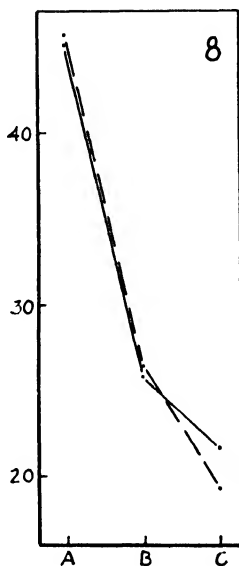
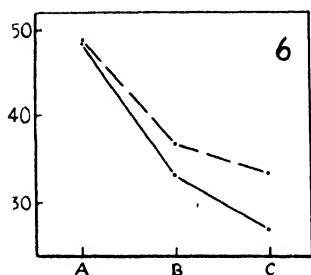
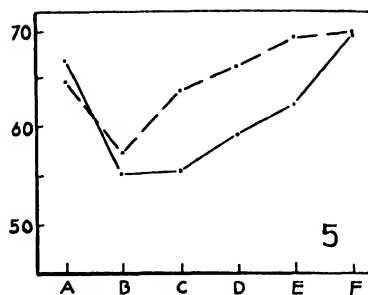
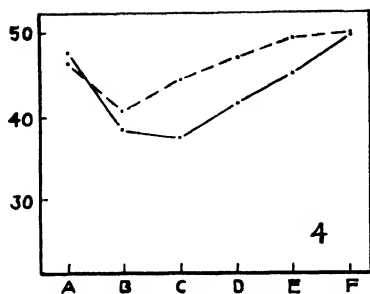
* P = probability of chance occurrence of difference as determined by method for standard error of the difference between means.

B. DESCRIPTION OF GROUPS BY METHOD II

	A		B		C	
	Mean	σ	Mean	σ	Mean	σ
Experimental...	+1.12	0.49	+0.15	0.48	-0.13	0.39
Control.....	+1.00	0.38	+0.15	0.36	-0.02	0.47

In either case the difference between the indices for experimental and control groups is so slight that it is not likely that any significance would be attached to it.

These data from Hinrichs have been subjected to further analysis. The differences between the means of the experimental and control groups at each of the three levels have been tested for significance by the test for the standard error of the difference between means. The pertinent data are recorded in Table 11, A. The probabilities of chance occurrence of such differences listed indicate that, on the basis of the older indices, the difference at the A level is possibly significant but that no significance can be attached to the differences between the experimental and control groups at levels



FIGS. 4 and 5.-Graphical presentation of data from Child (see text) on the effect of cyanide on head frequency of one-sixth pieces of *D. dorotocephala*. Solid lines represent the control pieces, and broken

B and C. Comparison between the means determined by the use of the indices of Method I of this paper indicates that none of the differences are significant, except that at the C level the difference ($P = 0.100$) may be suggestive of a trend.

The procedure developed by Wright (1934) and outlined in Method II of this paper has been applied to Hinrich's data, and the ranges of the three middle classes have been determined as in the proportion of $0.12:0.10:0.78$. Using these values for the theoretical class ranges, the means and their theoretical standard deviations have been calculated relative to a scale on which the threshold for anophthalmia is at 0 and for normal at +1. This information is tabulated in Table 11, B. While this procedure locates the means relative to each other and to the ranges of the three middle classes and gives a measure of the variability of the groups, it does not afford any reliable way of making further comparisons or statistical evaluations. The standard errors based on the derived standard deviations do not constitute an adequate basis for a statistical test. The method gives a description of the groups but no more. The values determined indicate for levels A and C the same relationship between experimental and control groups as that revealed above by the use of either scale of head indices, a relationship shown graphically in Figures 8 and 9. At level B the calculated means fall at the same point on the scale.

Hinrichs' conclusion in regard to this and similar experiments, that as a result of the experimental treatment "head frequency is lowered in C pieces and slightly raised in A pieces," has been made on the basis of the percentage distribution of head types without resort to any statistical treatment of the data. The analysis of the single experiment given above can be taken to indicate only that the single experiment is not conclusive but that the trends are in the direction Hinrichs has indicated. If the same trends are present in her other experiments as in the one studied here, it is probable that more complete analysis would give statistical evidence to substantiate her conclusion.

The curves presented in Figures 10 and 11 are based on unpublished data on the effect of aggregation on head formation in *D. dorotocephala*. The curves of Figure 10 are computed from the values of the equal-interval, head-frequency scale, and those of Figure 11 on the basis of the indices determined by Method I. The points along the

lines the experimental pieces. The levels of the body are indicated as abscissas; the head-frequency indices are located along the ordinates. Ordinates for Figure 4 are calculated on the basis of the equal-interval scale, those for Figure 5 on the basis of the indices determined by Method I.

FIGS. 6 and 7.—Graphical presentation of data from Buchanan (see text) on the effect of chloretone on head frequency of one-third pieces of *D. dorotocephala*. Solid lines represent the control pieces, and broken lines the experimental pieces. The levels of the body are indicated as abscissas; the head-frequency indices are located along the ordinates. Ordinates for Figure 6 are calculated on the basis of the equal-interval scale, those for Figure 7 on the basis of the indices determined by Method I.

FIGS. 8 and 9.—Graphical presentation of data from Hinrichs (see text) on the effect of caffeine on head frequency of one-third pieces of *D. dorotocephala*. Solid lines represent the control pieces, and broken lines the experimental pieces. The levels of the body are indicated as abscissas; the head-frequency indices are located along the ordinates. Ordinates for Figure 8 are calculated on the basis of the equal-interval scale, those for Figure 9 on the basis of the indices determined by Method I.

FIGS. 10 and 11.—Graphical presentation of data from the author's experiments on the effect of aggregation on head frequency of one-eighth pieces of *D. dorotocephala*. Ordinates represent head-frequency indices, and abscissas the levels of the body. Points along the broken line are averages for 10 experiments with pieces isolated in 5 ml.; points along the solid line are averages for the 10 paired experiments with pieces in groups of 10 in 50 ml. Ordinates for Figure 10 are calculated on the basis of the equal-interval scale, those for Figure 11 on the basis of the indices determined by Method I.

broken lines represent averages of the results of 10 separate experiments, each experiment consisting of 10 fragments from each of the 8 levels of the body indicated as abscissas, each fragment isolated in 5 ml. of water. Each of these experiments was

TABLE 12

EVALUATION OF DATA FROM AGGREGATION EXPERIMENTS DISCUSSED
IN TEXT AND PRESENTED IN FIGURES 10 AND 11

(*D. dorocephala*) (Ten paired experiments)

A. COMPARISON OF VALUES FOR MEANS OF ISOLATED AND GROUPED PIECES
DETERMINED BY THE USE OF HEAD-FREQUENCY INDICES

	A	B	C	D	E	F	G	H	Entire Series
Indices of equal-interval scale:									
Isolated mean	44 79	32 78	17 40	14 19	20 46	18 16	25 24	43 28
Group mean	44 11	25 08	13 74	13 21	18 07	16 62	24 23	43 56
$m_I - m_G$	0.68	7.70	3 66	0 98	2 39	1 54	1 01	- 0 28
P^*	0 260	0 0014	0 034	0 563	0 100	0 392	0 698	0 847	0 041
Indices of Method I:									
Isolated mean	62 78	52 28	40 70	38 43	43 14	41 46	46 78	62 92
Group mean	62 36	46 59	38 09	37 41	41 38	40 32	45 85	62 35
$m_I - m_G$	0 42	5 69	2 61	1 02	1 76	1 14	0 93	0 57
P	0.632	0 0058	0 040	0 444	0 100	0 444	0 629	0 701	0.023

* P = probability of chance occurrence of differences as determined by "Student's" method.

B. VALUES FOR MEANS AND STANDARD DEVIATIONS OF ISOLATED AND
GROUPED PIECES DETERMINED BY METHOD II

	A	B	C	D	E	F	G	H
Isolated mean	+0 98	+0 49	-0 14	-0 61	0 00	-0 12	+0 16	+1 02
Isolated σ	0 30	0 50	0 40	0 67	0 39	0 44	0 56	0 47
Grouped mean	+0 95	+0 13	-0 26	-1 20	-0 28	-0 20	+0 09	+1 05
Grouped σ	0 40	0 74	0 36	1 10	0 68	0 48	0 66	0 52
$m_I - m_G$	+0 03	+0 36	+0 12	+0 59	+0 28	+0 08	+0 07	-0 03

Comparison of means of entire series by "Student's" method

$$P = 0.035$$

paired with an experiment set up at the same time in which the 10 fragments from each level were grouped in 50 ml. of water. The averages for these 10 experiments with grouped pieces are indicated by the points along the solid lines.²

² The result tabulated here appears to indicate that in this particular series of experiments and with the volumes used the isolated pieces formed better heads than grouped pieces. This conclusion is not to be taken as representative of all the experiments, for in other combinations of volumes the grouped pieces were at times favored.

The choice of the scale of indices makes little apparent difference in the relationship revealed between the isolated pieces and the grouped pieces, as is indicated by comparing Figures 10 and 11. The indices of Method I bring about a reversal of the points at level H and bring the trend at that level into conformity with the trends at each of the other seven levels of the body. Since the points represent averages for paired experiments, it is possible by the use of "Student's" method ("Student," 1925) to determine the significance of the differences indicated. The *P* values for these tests are shown in Table 12, A. With either scale of indices it appears that the differences at levels B and C are probably significant. At levels A, D, E, F, and G the isolated pieces are favored, but in no case is the difference a significant one. Since the differences at level H are of no significance, the reversal of the relationship between isolated and grouped pieces is of no consequence, although the situation indicated by the use of the indices of Method I seems more plausible since the isolated pieces are favored at the other 7 levels. Confidence is felt in the application of the indices of Method I to these particular data, since the indices are developed on the basis of the observed frequency distribution of head types in these experiments and in others performed on worms from the same stock.

In a case like this one it is perhaps helpful to have some test to express the result of the whole series of experiments rather than the separate effect at each of the eight levels. By comparing the means of isolated and grouped pieces at each of the eight levels, it is possible now to test the significance of the entire series by regarding the means of the values for isolated and grouped pieces at each level as a pair of values and testing the series of 8 pairs by "Student's" method. The *P* values for these comparisons are shown in the final column in Table 12, A. They indicate that, when the results are calculated by either of the sets of indices used, the isolated pieces have formed heads which are probably significantly better than those formed by grouped pieces.

For this same series of experiments on isolated and grouped pieces Method II has been used to determine the means and standard deviations of the sixteen separate populations relative to the ranges of the three intermediate classes of head types. This information is tabulated in Table 12, B. It shows for the levels A-G, inclusive, that each mean for isolated fragments lies above the mean for its respective control of grouped pieces. At level H the mean for the grouped pieces is slightly above that for the isolated pieces, a difference also indicated when the results are calculated by the older (10-50) scale of indices. The average of the differences between the means (comparing at each level the mean for isolated pieces with the mean for grouped pieces) is ± 0.15 . The probability that such a difference is due to chance is 0.035 ("Student's" method). This confirms the conclusion arrived at by assigning indices, i.e., that isolated pieces have formed better heads than grouped pieces in this particular set of experiments.

It would be possible to make further comparisons of the data from these experiments by throwing together the result of all 10 experiments with isolated pieces and comparing it with the total of the experiments on grouped pieces. Comparisons could then be made for the eight levels by use of the method for the standard error of the difference between means. There is nothing to be gained by such a comparison. "Student's" method has a distinct advantage for the analysis, since it takes into account the pairing of the experiments and since each experiment is compared directly with a control experiment set up at the same time.

In general, any individual curve drawn by use of the indices developed in Method I

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is little different from one drawn by use of the older indices. This has been sufficiently shown in the cases already discussed and illustrated. When applied to isolated experiments, in which small numbers of individuals have been used, a reversal of the slope of a small portion of the curve may be effected. An example is given in Figure 12, the result of a single experiment with 10 worms (10 pieces at each of the eight levels). The solid line represents a plotting of the head-frequency data on the basis of the indices of the 10-50 scale, and the broken line the same data computed on the basis of the 35-70 scale of Method I. The slope of the curve between levels F and G is reversed by the change in the scale of indices. Such a reversal consistently occurring might demand

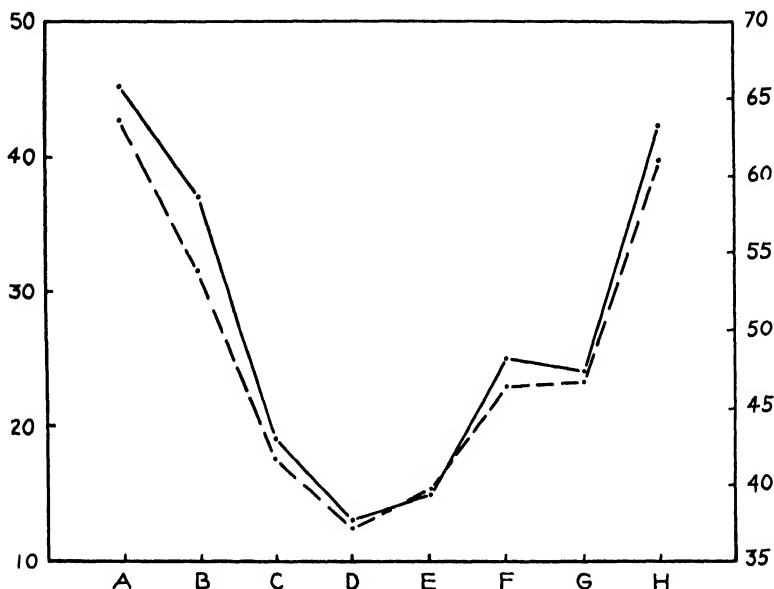


FIG. 12. —Graphical presentation of data from a single experiment with *D. dorotocephala*; one-eighth pieces, 10 from each level, each piece isolated in 1 ml. of water. The levels of the body are indicated as abscissas. Ordinates for the points along the solid line are calculated on the basis of the equal-interval scale (on left) and for the points along the broken line on the basis of the indices are determined by Method I (on right).

a reinterpretation of head-frequency phenomena as related to the axis of the worm. It appears, however, that the reversal is the result of the small number of cases and of peculiarities of the particular data used. In averages of experiments or in experiments where considerable numbers of pieces are involved, such changes in slope have not been observed to occur. In general, a change to the revised scale of indices results in a "smoothing" of the curve but in no great change in its general character.

DISCUSSION

The methods presented in the preceding pages represent an attempt at more accurate expression and evaluation of head-frequency data from experiments with planarians. By the procedure outlined in Method I definite values are assigned to the different

types of heads and are based upon the observed frequency of occurrence of those types. Its application depends upon the availability of data from experiments in which the experimental groups are small, in order that many units may be selected, each of which shows the whole range of head types. It appears appropriate to apply such a method to the evaluation of data from regeneration experiments in which small numbers of pieces must of necessity be used. When each of these experiments is paired with an experiment set up at the same time and the paired experiments are repeated a number of times, the significance of the values determined may then be tested by a statistical method such as that presented by "Student" (1925).

Method II of this paper, developed by Wright, is one more suitable for expressing the results of experiments in which large numbers of pieces have been used and the experiments have not been replicated. It depends for its application upon the determination of the ranges for the three middle classes of the head-type scale. The variability of the regenerated structures can then be described, and the mean of the group located with respect to the ranges of the three middle categories.

Sufficient evidence has been presented to demonstrate that the head indices of Method I and the class ranges of Method II are not constants to be used in all work with a particular species. Wright determined from the combined data of three separate workers that the ranges of the three intermediate head classes were in the proportion of 0.36:0.14:0.50. The application of Method II to Hinrichs' data in a preceding section revealed that in her experiments the ranges of the same three categories were in a proportion of 0.12:0.10:0.78. The ranges determined by the application of this method to the present author's data are in the proportion of 0.19:0.12:0.69. This proportion is almost identical to that arrived at by Method I from a study of selected experimental units showing the whole range of head types (0.18:0.13:0.69). The similarity of the two proportions last cited, determined by different methods from the same data, confirms the adequacy of Method I for the determination of these ranges.

No attempt will be made to reconcile the differences in distribution of head types brought out above. Differences in the duration of the experiments considered or differences in the classification of regenerated heads may be sufficient to account for a considerable discrepancy. This more or less subjective error may be introduced at either end of the head-frequency scale. Disagreement between workers on the degree of disparity in size of the eyes necessary to cause a head to be rated as teratophthalmic rather than normal would account for a shift in the threshold between normal and teratophthalmia. A shift at this end of the scale may be caused by experiments of different length, for it has been observed by some workers that, if teratophthalmic pieces are followed over a period of time, some regulation of the eye size may take place, and eyes once classified as unequal in size may later be considered as of normal proportions. At the lower end of the scale, workers may disagree in their evaluation of the amount of regenerated tissue necessary to constitute an anophthalmic outgrowth, or, if allowed to reconstitute over a period of time, an appreciable number of pieces once classified as acephalic will develop anophthalmic outgrowths. Either of these events will cause an apparent shift in the position of the threshold between acephaly and anophthalmia. It is, in addition, probable that the stocks used by different workers differ not only in their head-forming ability (Rulon, 1938, p. 210) but also in the ranges of the head classes along the head-frequency scale. These considerations make it imperative that application of either of the new methods of evaluation presented in this paper should

not be undertaken unless the ranges of the classes of heads are first determined for the particular stock of worms being used. Unless this is done, neither scheme appears to have any distinct advantage over the scale of indices that has long been used.

There is no available measure of the success of planarian regeneration other than the classification of the head types into visually recognizable categories. The differences brought out between different stocks and the observations of different workers in classifying head types do not imply that this classification is an unreliable method for the evaluation of head frequency. The differences do, however, indicate that caution must be exercised in comparing the observations of different workers and of the same or different workers when the observations are based on experiments in which the duration of the period of regeneration is not constant or worms from different stocks are used.

It is assumed that for a given stock the experimental treatment will not greatly modify the values for the indices or for the ranges of the head classes. This assumption may not be strictly true if the experimental treatment is severe and the differences in head frequency between experimental and control groups are great. It is necessary, however, to proceed on such an assumption in order to have a common basis for comparison. If the differences are so great that they cause great changes in the indices or class ranges, it is likely that the result of the experiment will be sufficiently clear without the necessity for more complete analysis of the data.

Exception may be taken to the choice of the class medians as indices in Method I of this paper. If the populations being compared differ greatly from each other, the positions of the medians will be inconstant. The values for the intermediate groups will remain fairly constant relative to each other, so long as these groups are located near the mean for the entire population being considered. Median values for the extreme classes (normal and acephalic) will be most inconstant if the numbers of individuals in the extreme classes are small and if the two populations differ greatly in their central tendency. For use in comparing results from experiments in which the populations differ greatly in their distribution the mid-point of the range for each class might be more representative. As long as the intermediate classes are small and near the mean of the entire range, the values for the mid-points of those classes will not differ greatly from those for their medians. For the two extreme classes the medians and mid-points will be farther separated. The choice in this case is purely arbitrary. Since the indices are intended for strict application only in experiments on relatively homogeneous stocks for which the indices have been determined and since the extreme classes are both large, it is thought that no serious error will be introduced by the use of the medians. The essential feature is not the choice of the value but rather its consistent application. The use of these definite values for the head types possesses a real advantage in the expression of experimental results, and, as has been pointed out, these indices will be more critical than the arbitrarily assigned values long used if they are determined on the basis of the observed frequencies of occurrence of the head types.

No account has been taken of the factors involved in the determination of the type of head. These factors may be intrinsic or extrinsic and have been rather fully analyzed in the papers of Child and of his students (Child, 1912, 1914, 1916, 1920; Child and Watanabe, 1935; Buchanan, 1922, 1923; Rulon, 1936, 1937; etc.). Given carefully controlled external conditions to which the fragments are subjected, some variation in the type of head formed is to be expected as a consequence of inequalities in the size of

fragments or differences in the physiologic condition of the individual worms from which the fragments have been isolated. Such differences are sufficient to account for the formation of heads of all types in small groups of pieces. Similarly, minute and uncontrollable differences in the conditions in the immediate vicinity of fragments placed even in the same container may be sufficient to account for variations in the types of heads regenerated. Indeed, without variations due to one or the other of these types of factors such an analysis as is here attempted could not be made. In the experiments used as the basis for these observations it seems most likely that the primary causes for lack of uniformity within the units of 10 pieces may be those traceable to differences in size of piece or physiologic condition of the individual worms. These differences are impossible to control, even though all pieces are cut by the same person and the 10 worms used in each experiment are from the same source and have had identical histories of laboratory culture. Regardless of the cause of the variation observed, the method of analysis would remain the same.

The differences observed in the calculated values of Method I to be used in estimating the head frequency in the two species of *Dugesia* here considered are perhaps to be anticipated. There is no a priori reason for expecting that the distribution of head types will be the same for all species. Indeed, the estimations of class ranges already made for different data from *D. dorocephala* are indicative of differences within a species. Such a difference also has been observed in the author's experience with *D. tigrina*. The data already presented for this species are based on experiments with worms from well-known collecting grounds in the town of Falmouth, Massachusetts, and from a stream near East Providence, Rhode Island. Worms from these localities appear to be similar in life-history and behave similarly in regeneration. A few experiments with worms collected from Mary's Lake on Naushon Island near Woods Hole, Massachusetts, have yielded data which indicate that they behave differently in regeneration. As has been pointed out by Curtis (1902) and Kenk (1937), worms collected from this locality have never been observed to pass through a sexual phase in their life-history. Because of this failure to exhibit reproductive organs, their identification remains uncertain, although they are believed to constitute a race or variety of *D. tigrina*. The distribution of head types observed in this race is very different from that recorded in Table 2. It indicates that in this species as in *D. dorocephala* no one scale of indices or estimate of class ranges is satisfactory for application to all data obtained from work done with the particular species.

It is not to be inferred that the originators and users of the old scale of indices intended it to be more than an approximation for convenience. Indeed, Buchanan has stated (1923, p. 410) that "no claim is made that the values 5, 4, 3, 2, 1, represent any exact mathematical valuation of regenerated tissue; this is simply a convenient and consistent way of showing the effects of the agent on head frequency." For the purpose designed, the old scale of equal intervals appears in most cases to have been entirely adequate for outlining major differences in head frequency along the axis of the planarian body or for distinguishing between the head frequencies of experimental and control groups where the differences are not obscure. Where such differences are slight, however, the use of a scale in which the indices or class ranges do closely approach the values based on observed frequencies of occurrence of the regenerated structures probably comes closer to revealing the real relationships between control and experimental groups.

SUMMARY

1. Methods are presented for the determination of the class ranges, limits, and medians for the different types of anterior regenerated structures in planarians. These values are based upon the observed frequencies of occurrence of the five recognizable head types.

2. The chosen indices of Method I are the abscissas of the medians of the different classes, located relative to a scale of 0-100 embracing the entire range of variability of head types. The determination of these indices is made on the basis of the percentage distribution of head types in many small, experimental units, each unit showing the whole range of the classes of head types. The indices appear most suitable for use in the evaluation of data from experiments in which the numbers of pieces in each experiment are of necessity small.

3. By Method II, an application of a method developed by Wright, the theoretical ranges of the three intermediate classes of heads are determined by statistical means from data from experiments with large numbers of pieces from a homogeneous stock. The mean for any particular population of regenerated structures may then be located relative to the ranges of the three intermediate classes and its variability described in terms of a derived standard deviation. Application of the method may be made to experiments in which the indices of Method I could not be determined from the available data.

4. Cases chosen for illustration demonstrate the application of different available methods to the evaluation of head-frequency data. Use of the revised indices of Method I may bring about an apparent reversal of the relationship between experimental and control groups, as compared to the same data evaluated by the indices of the equal-interval scale of previous workers. The availability of several methods makes possible the use of more than one method for the evaluation of data. When followed by a statistical test, a comparison of the results of application of more than one method will confirm the significance of experiments the outcome of which is in doubt.

5. Differences brought out in the distribution of head types formed by different species or by planarians from different stocks within a species or as determined by different workers make it essential that any indices or estimates of ranges intended for strict application must be determined for the particular body of data being evaluated.

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A MIGRATION-DILUTION APPARATUS FOR THE STERILIZATION OF PROTOZOA¹

(One plate and one figure)

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I. INTRODUCTION

PROTOZOA may be divided roughly into large groups depending upon how they respond to various influences. One such large group is negatively geotropic. The apparatus described herein has been designed to separate this type of protozoa from their associated bacteria, provided the protozoa will behave in a negatively geotropic manner in a closed vessel.

Many different techniques for the sterilization of protozoa have been employed in the past, and, while it has been demonstrated that it is possible to obtain sterile organisms by these methods, they all have very serious limitations. Hargitt and Fray (1917) and Parpart (1928) used methods employing a dilution factor as the predominant means for effecting sterilization by washing in Petri dishes. Cleveland (1928) made use of lateral migration causing protozoa to migrate across a 10-inch Petri dish. Hetherington (1934) combined both migration and dilution in a series of washes and lateral migrations in depression slides inclosed in Petri dishes. The chance for contamination due to manipulations inherent in these methods is considerable, because large areas of fluid surface are exposed to air contamination every time an organism is transferred into or out of these dishes. Moreover, a considerable amount of time and work is necessary to sterilize one organism. Glaser and Coria (1930) caused organisms to migrate up a tube, but here the dilution factor is usually not great enough to take care of all the associated bacteria, particularly if by chance *Pseudomonas* or other rapidly moving bacteria are present in the culture.

The centrifuge method (Kidder and Stuart, 1939), employing at least fifteen washes, is limited in its application to situations where the investigator has a high concentration of protozoa to start with. The chances of contamination through faulty manipulative technique are extremely great in this method, involving as it does at least fifteen transfers from one sterile centrifuge tube to another by means of sterile pipettes.

In an attempt to overcome these limitations an apparatus was designed incorporating both a migration and a dilution factor in a closed system. The organisms are introduced at one end of the system and are not exposed to any chance contamination until they are taken away from the apparatus after the final migration. The dilution factor is of the order of 10^6 , and the amount of material recovered in a sterile condition is as great as or greater than any method heretofore described. The time necessary for the protozoa to complete the migration through the apparatus varies roughly from 20 minutes to 1 hour depending on the type of protozoa.

¹ I wish to acknowledge the timely suggestions of Professor G. W. Kidder. His constructive criticisms and his painstaking tests of this apparatus have aided greatly in its development.

II. APPARATUS AND METHOD

The apparatus consists of a series of six flasks,² 8 inches tall, each holding approximately 145 ml. of fluid, joined together so that the top of one flask is connected by small-bore rubber tubing to the bottom of the next succeeding flask (Fig. 1). The rubber tubing is securely fastened to the glassware. A reservoir (capacity 1 liter) capable of being raised to a level higher than the flasks is joined to the bottom of flask No. 1 by rubber tubing. A test tube into which a glass tube is inserted through a cotton plug is connected by rubber tubing to the top of flask No. 6. The fluid in the raised reservoir is held in check by a spring clamp. By releasing the clamp it is possible to transfer 1.5 ml. of fluid from the reservoir into the bottom of flask No. 1. This effects a transfer of the uppermost 1.5 ml. of fluid from each flask into the bottom of the next succeeding flask in line. The uppermost 1.5 ml. of fluid in flask No. 6 is at the same time transferred into the test tube. A vaccine port is blown into the side of flask No. 1 near the bottom (Fig. 1), to which is fitted a vaccine cap. Through this cap the protozoa to be sterilized are injected with a sterile hypodermic needle.

The migration flasks and reservoir are mounted on a brass plate by means of cast-bronze rings held by brass posts (Pl. I). The cast-bronze rings are held against the glassware by light coiled springs which allow the glassware to expand during autoclaving.

The reservoir is assembled in a brass cradle capable of being raised and lowered on a brass rod firmly attached to the brass plate. It is lowered so that the entire apparatus will fit into the 10-inch door of a small autoclave. The brass plate, with the assembled flasks and reservoir, rests on, but is separable from, a wooden base 2 inches high which carries a suitable clamp for holding the final sterile test tube.

The apparatus is partially filled with fluid and sterilized. This fluid may be slightly alkaline distilled water or any other nonnutritive or nutritive media.

With the reservoir in the lowered position the fluid is adjusted in the flasks by means of an aspirator until flask No. 6 is empty and there is about 1 inch of fluid in flask No. 5 and the reservoir.

An auxiliary 1,000-ml. flask is three-quarters filled with the same fluid that is to be used in the migration apparatus, cotton plugged, and autoclaved with the apparatus, together with the following:

- 12 empty, cotton-plugged test tubes
- 1 No. 18 hypodermic needle in cotton-plugged serological tube
- 1 large 50-ml., cotton-plugged centrifuge tube
- 1 rack of micropipettes fitted with cotton plugs, each in its own serological tube
- 50 test tubes each filled with 2 ml. of some suitable fluid medium in which to culture the sterile organisms obtained

All these are autoclaved at 15 pounds' pressure for 20 minutes. This should be done at least 12 hours before the apparatus is to be used, in order to allow it to cool to room temperature and establish equilibrium to avoid convection currents. A 5-ml. glass syringe to be used for the injection of the protozoa to be sterilized is boiled for 20 minutes.

Organisms are sterilized as follows: The apparatus is placed on the wooden base, and the test tube is placed in the clamp provided for it so that it rests at table level below the level of the migration flasks. The wire clamp is tightened on the rubber tubing connecting the reservoir to flask No. 1. The reservoir is raised to the higher level and is then filled

² These may be obtained from Charles Kaziun, 338 Winter Street, Brockton, Massachusetts.

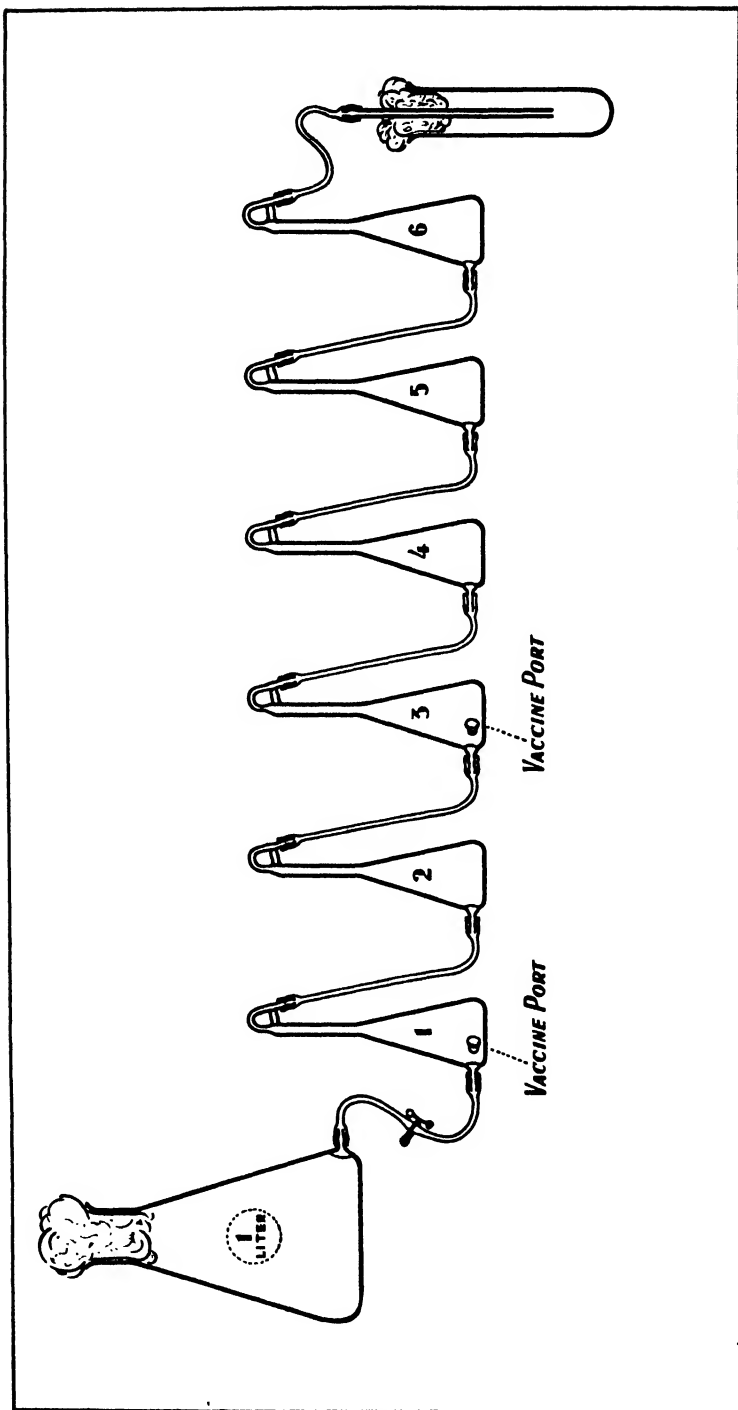


FIG. 1.—Diagram of migration-dilution apparatus drawn to show connections

with fluid from the auxiliary flask by removing both cotton plugs, flaming the necks, and pouring the liquid quickly from the auxiliary vessel into the reservoir, while holding a Bunsen burner so that the flame protects the point of operation from air contamination. The reservoir is again cotton plugged. The spring clamp is loosened, and the fluid is allowed to flow down into the migration flasks until they are completely filled, together with the tubing coming from flask No. 6. This rids the system of any air pockets. About 3 ml. of fluid are allowed to run into the test tube. The flow of liquid is now checked, and a dry sterile test tube is substituted for the tube containing the 3 ml. of fluid. The sterile dry cotton plug taken from the dry test tube is used to plug the tube taken from the apparatus, and the cotton plug, through which the glass "take-off" tube is passed, is used, of course, to plug the dry tube.

The protozoa to be sterilized are concentrated by centrifugation and, if the contamination is heavy, may be washed a few times by this method (Kidder and Stuart, 1939). This removes the debris and larger clumps of bacteria. The supernatant fluid (i.e., all but 2 ml.) is removed with a sterile 10-ml. pipette, and the cotton plug is replaced. A fresh sterile 10-ml. pipette is used to transfer the organisms in the remaining 2 ml. of fluid (less the very tip fluid) to the 50-ml. centrifuge tube. The protozoa are transferred from the 50-ml. centrifuge tube through the vaccine port to the bottom of flask No. 1 by a sterile hypodermic needle and syringe. The protozoa start to migrate up flask No. 1 (migration takes from 1 to 8 min. depending on the organisms used and their physiological state at the moment). The migration may be followed with a $5\times$ – $10\times$ hand lens.

When sufficient numbers have accumulated at the top of flask No. 1, the spring clamp is released, and about 1.5 ml. of fluid is allowed to flow through. This may be gauged by the amount which flows into the test tube from flask No. 6. This operation deposits the protozoa in the bottom of flask No. 2, and they start to migrate to the top of this flask. The test tube is now replaced with a sterile dry one. When the protozoa are in sufficient numbers at the top of the second flask, they are transferred as above to the third flask. The first five test tubes taken off in this manner serve as controls for the sterility of the apparatus, inasmuch as the fluid from each may be poured on agar plates. We have never found a contaminated plate from this source.

The sixth and following test tubes taken off should contain the protozoa; and 0.5 ml. of the contents are taken off with a sterile pipette and deposited in a sterile Petri dish placed on the stage of a dissecting microscope, fitted with a Plastacele (Dupont) drape. The field is immediately searched, and single organisms are transferred by means of small sterile micropipettes to shell vials or test tubes containing either sterile nutrient media or sterile distilled water. If the protozoa are obligate bacteria-feeders, these vials have been previously bacterized with a light suspension of a pure strain of a favorable bacteria.

The following precautions are necessary for the successful operation of the apparatus.

Flask No. 6 should be empty, and there should be about 1 inch of fluid in flask No. 5 when the apparatus is autoclaved. This allows for expansion of fluid during autoclaving. If more fluid than this is allowed to remain in the system, it will flow over into the test tube, fill it, and soak the cotton plug.

The apparatus should be autoclaved without the spring clamp on the rubber tubing coming from the reservoir. Failure to do this will result in a blown-out rubber tube.

After the reservoir has been filled from the auxiliary flask, enough fluid should be allowed to flow through the apparatus to fill it completely, including the tube coming from the top of flask No. 6, thereby expelling all air bubbles from the system.

When injecting flask No. 1 with the protozoa, care should be exercised so that no air is introduced into the system with the protozoa. This is of extreme importance. Should an air bubble be introduced, it would rise rapidly in the flask and might destroy the sterility of the fluid therein and would travel ahead of the migration of the protozoa throughout the system carrying possible contamination with it.

The transfer of the glass take-off tube from one sterile test tube to another should be done with aseptic technique, being careful not to allow the glass tubing to touch the sides of the test tube.

Bacteria-feeding forms which encyst readily (*Tillina*, *Colpoda*, etc.) may be recovered in a pure bacteria-protozoa culture by the following modification:

Flask No. 3 is provided with a vaccine cap. The cap is covered with cotton and soaked with 70 per cent alcohol for at least 20 minutes before injection is made. As soon as the protozoa have completed the migration through the second flask and have been carried over into the bottom of the third flask, the fluid of this flask is supplied with a suspension of the food bacteria, injected through the vaccine cap.

The subsequent technique is as already outlined.

Flasks No. 1 and No. 6 may each be provided with an additional vaccine cap through which a platinum wire is inserted. A bead of glass bonded to the wire on the inside of the vaccine cap keeps the wire from being pushed out by pressure during autoclaving. These wires provide the means for passing an electric current through the fluid in the apparatus during migration, if the investigator wishes to study the effects of electric current as an aid to migration.

III. EXPERIMENTAL EVIDENCE AND QUANTITATIVE BACTERIOLOGICAL TESTS FOR STERILITY

Paramecium caudatum were isolated from a wild environment and fed for 8 days through 2 transplants on live yeast. The culture was concentrated and put through the apparatus. Migration was very slow, but one *Paramecium* was recovered and placed in a tube of proteose-peptone broth. This tube was incubated for 3 weeks at 26° C. The broth remained clear and at the end of this period was flooded onto agar plates, and the plates remained entirely free of bacterial growth.

*Tillina canalifera*³ were isolated from a hay infusion and grown on *Vibrio* sp. in association with other bacterial contaminants. One milliliter of this culture, concentrated by centrifugation, was injected into the apparatus. Very rapid migration took place. The entire migration consumed only 25 minutes. From 50 to 100 ciliates were recovered in tube No. 6, and about 50 organisms each in tubes Nos. 7, 8, 9, and 10.

Single organisms were not isolated, but the whole tube was bacterized with *Vibrio* sp. After considerable growth (48 hr.) 9 agar plates were streaked from tube No. 6, and 3 plates each were streaked from tubes Nos. 7, 8, 9, and 10.

Tube No. 6 showed only typical *Vibrio* sp. after a 10-day incubation period. Therefore, this is considered an adequate method for obtaining this ciliate in a pure bacteria-protozoa culture. Small numbers of extraneous bacteria appeared on the plates from tubes Nos. 7, 8, 9, and 10, however, and these tubes were discarded.

Many migrations of *T. canalifera* were made, and it was observed that the success of the migration was dependent on their physiological condition. If too well fed, they did

³ This strain of *Tillina* was sent to Professor Kidder in 1937 through the courtesy of Dr. J. P. Turner and has been maintained in this laboratory ever since.

not migrate but formed division cysts on the sides of the flasks; if starved, they formed permanent cysts. Migration results were the best when the organisms used were within 4 or 5 hours of the time they normally would form protective cysts. They appeared to be most active at this period.

Tetrahymena geleii (Furgason, 1940) growing in a wild culture with heavy bacterial growth were concentrated by centrifugation and 1 ml. was introduced into flask No. 1. The migration fluid was 0.5 per cent yeast extract. One milliliter was taken over at each transfer of ciliates from one flask to the next in line. Ten tubes were collected. Migration was excellent and consumed 30 minutes.

The results are tabulated in Table 1.

TABLE 1
Tetrahymena geleii
(Whole-Tube Turbidity Test)

Tube No.	Ciliates Present	Sterile at 48 Hours	Sterile at 72 Hours	Tube No.	Ciliates Present	Sterile at 48 Hours	Sterile at 72 Hours
1.....	o	+	+	6.....	+	+	+
2.....	o	+	+	7.....	++	+	-
3.....	o	+	+	8.....	+++	-	-
4.....	o	+	+	9.....	+++	-	-
5.....	o	+	+	10.....	+++	-	-

Six organisms were isolated from tube No. 6, 14 from tube No. 7, and 5 from tube No. 8. These were set up in shell vials containing 0.5 per cent sterile yeast extract.

Results are tabulated in Table 2. All plates (Table 2) were prepared in duplicate series, one set incubated at room temperature, and the other at 37°C.

TABLE 2
Tetrahymena geleii

Vial Number	From Tube Number	No Turbidity at 96 Hours	Plates Sterile at 96 Hours	Plates Sterile at 240 Hours	Replatings Sterile at 240 Hours	Vial Number	From Tube Number	No Turbidity at 96 Hours	Plates Sterile at 96 Hours	Plates Sterile at 240 Hours	Replatings Sterile at 240 Hours
1.....	6	+	-	-	-	15.....	7	+	+	+	+
2.....	6	+	+	+	+	16.....	7	+	-	-	-
3.....	6	+	+	+	+	17.....	7	-	-	-	-
4.....	6	-	-	-	-	18.....	7	+	-	-	-
5.....	6	+	+	+	+	19.....	7	-	-	-	-
6.....	6	-	-	-	-	20.....	7	+	-	-	-
7.....	7	+	-	-	-	21.....	8	+	+	+	+
8.....	7	+	+	+	+	22.....	8	-	-	-	-
9.....	7	-	-	-	-	23.....	8	+	-	-	-
10.....	7	+	+	+	+	24.....	8	-	-	-	-
11.....	7	+	-	-	-	25.....	8	-	-	-	-
12.....	7	+	-	-	-						
13.....	7	+	-	-	-						
14.....	7	+	+	+	+	% sterile*	68	32	32	32

* According to test.

This experiment shows how unreliable turbidity tests (Table 1) for sterility can be. Judging by turbidity, 68 per cent of the selected organisms appeared to be sterile, but agar plates streaked with the broth brought the percentage down to 32 per cent. Transplants from the 8 sterile tubes were heated (after 48 hr. of growth) to 40° C. for 5 minutes to kill the ciliates. These tubes proved to be entirely devoid of living organisms after 3 weeks of incubation at room temperature.

Glaucoma scintillans were concentrated from a wild culture, heavily contaminated with bacteria. The organisms were migrated through 0.5 per cent yeast extract in the apparatus. Tubes Nos. 6, 7, 8, 9, and 10 were collected. No ciliates were found in tube No. 6.

TABLE 3
Glaucoma scintillans
(Single Isolations from Tube No. 7)

TUBE No.	CILIA- TES OB- SERVED AFTER 70 HOURS	CILIA- TES PRESENT AFTER 127 HOURS	TURBID- ITY TESTS	AGAR PLATES			TUBE No.	CILIA- TES OB- SERVED AFTER 70 HOURS	CILIA- TES PRESENT AFTER 127 HOURS	TURBID- ITY TESTS	AGAR PLATES		
				Sterile after 70 Hours	Sterile after 127 Hours	Sterile after 240 Hours					Sterile after 70 Hours	Sterile after 127 Hours	Sterile after 240 Hours
1.....	—	—	+	+	+	+	15.....	+	+	+	+	+	+
2.....	—	+	+	+	+	+	16.....	+	+	+	+	+	+
3.....	—	+	+	+	+	+	17.....	+	+	+	+	+	+
4.....	+	+	+	+	+	+	18.....	+	+	—	—	—	—
5.....	+	+	+	+	+	+	19.....	—	+	+	+	+	+
6.....	—	+	+	+	+	+	20.....	—	—	+	+	+	+
7.....	+	+	+	+	+	+	21.....	—	—	+	+	+	+
8.....	+	+	+	+	+	+	22.....	+	+	+	+	+	+
9.....	+	+	+	+	+	+	23.....	—	—	+	+	+	+
10.....	—	—	+	+	+	+	24.....	—	+	+	+	+	+
11.....	—	—	+	+	+	+	25.....	—	+	+	+	+	+
12.....	—	—	+	+	+	+							
13.....	—	—	+	+	+	+	% ster-				96	96	96
14.....	+	+	+	+	+	+	ile..						

Twenty-five ciliates were isolated from tube No. 7 and placed one each in test tubes containing 0.5 per cent yeast extract. No isolations were made from tubes Nos. 8, 9, and 10.

Sixty-five per cent of the selected organisms started thriving⁴ cultures, and of these cultures 96 per cent proved to be sterile by turbidity and agar-plate tests. It will be noted here that the turbidity test agrees exactly with the agar-plate tests, whereas in the tests made on *T. geleii* there was marked disagreement. The fact that the division rate of *T. geleii* was much more rapid than *G. scintillans* under the conditions of this experiment probably supplies the answer. In a rapidly growing culture the bacteria may be consumed, so that turbidity tests are misleading. On the other hand, in slow-growing cultures turbidity tests usually are much more reliable. Table 3 shows a considerable

⁴ The method of culturing this species, together with its food requirements, will be reported later by Professor Kidder.

lag period for some of the tubes (ciliates were not observed even after 79 hr. but showed up at the end of 127 hr. in numbers sufficient to be observed); and so, if bacteria were present, conditions were very favorable for their rapid growth. The turbidity tests in this case proved to be equally as reliable as the agar-plate test for sterility.

IV. CONCLUSION

The apparatus and method herein described are not of universal application but have been found adequate for rapidly swimming forms which will rise in a vessel. With further study of various types of protozoa, pH of media, and possible application of electric current during migration it is believed this method will prove useful for a large number of similar species.

As already pointed out, the advantages of such a method are relative simplicity, lack of chance contamination during migration, short time of operation, and number of sterilized organisms recovered.

V. SUMMARY

1. An apparatus for sterilizing certain protozoa is described.
2. The apparatus takes advantage of both the migration of the protozoa through a sterile fluid and a high dilution factor.
3. Experimental data on four species of ciliates sterilized by this method are given.

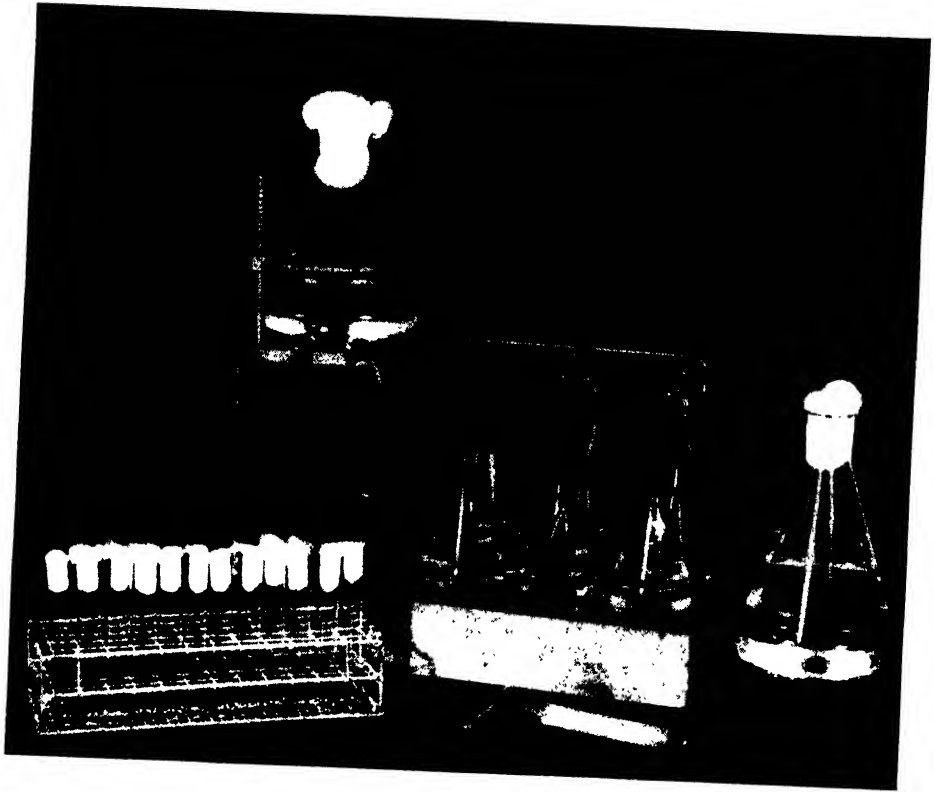
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PLATE I

Photograph of assembled migration-dilution apparatus

PLATE I



THE CRUSTACEAN SINUS GLAND AND CHROMATOPHORE ACTIVATION¹

(Ten figures)

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IT IS astonishing that so much work has been done upon the effects of eyestalk extract upon chromatophore response and yet the point of origin of the active substance in the stalk has not been determined conclusively. Although a recently published textbook of general physiology states the optic nerve to be the source of the substance, the blood gland (sinus gland) of the stalk has been most generally assumed to be the source since Hanström's (1935) experiments in this regard. In these experiments Hanström divided the eyestalks of various crustaceans transversely and found that the portion yielding chromatophorotropic activity invariably contained, among other things, the sinus gland. This evidence, although moderately convincing, was not conclusive. A nerve tissue, for instance, closely associated with the gland, could well have been the actual source.

Experiments have been performed which demonstrate very clearly the source of chromatophorotropic material in the eyestalk. A minute fragment of glandular tissue, about a hundredth part of the volume of the whole stalk which shows approximately 80 per cent of the chromatophorotropic activity of the whole stalk, has been isolated. Injection of an extract of this minute gland and implantation of the gland into animals from which the gland (eyestalk) had previously been removed give conclusive evidence of its activity.

The gland, from its position in the eyestalk and from the brief histological work that was done upon it (Brown and Cunningham, 1939), appears to be identical with Hanström's (1937) *Sinusdrüse* (blood gland) and is the source of the viability and molt-controlling activity described by Brown (1938) and confirmed by Brown and Cunningham (1939). Hanström's description of the gland in *Cambarus* was so inadequately done that definite identification has been difficult. It seems quite safe, however, to assume their identity and to speak of the gland in the following description as the sinus gland.

THE SINUS GLANDS OF SEVEN CRUSTACEANS

The sinus glands of each of the crustaceans so far examined (*Callinectes*, *Carcinus*, *Libinia*, *Pagurus*, *Uca*, *Crago*, and *Palaemonetes*) have been easily isolated from the remaining stalk tissue. The gland is quite conspicuous through the transparent exoskeleton of *Crago* and *Palaemonetes*, especially when the normal animal is placed in a strong beam of light and against a black background. In these latter forms it appears in the position indicated in Figure 1, *B* and *F*, as a relatively opaque mass of bluish-white

¹ This investigation was supported in part by a research grant from the Graduate School of Northwestern University.

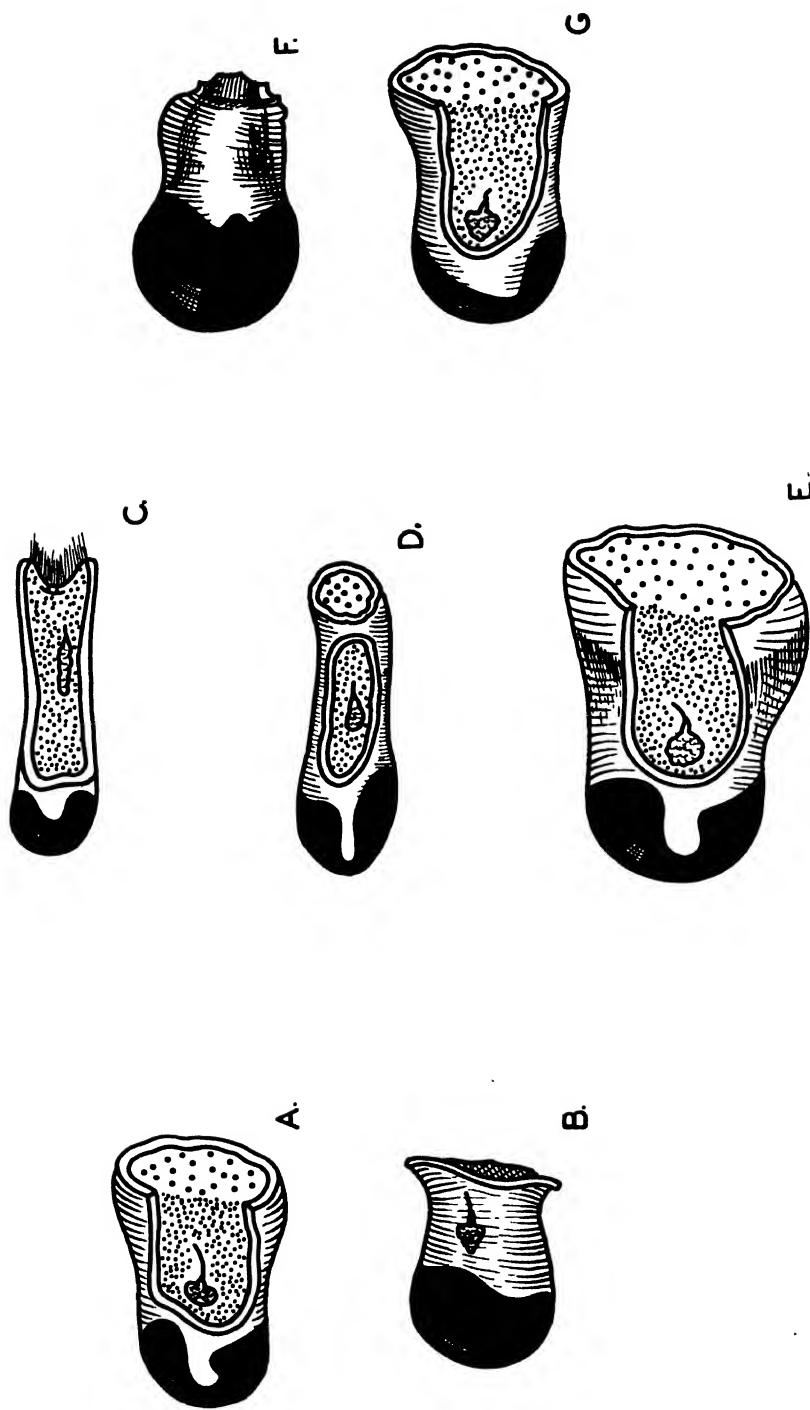


FIG. 1.—The left eyestalks of 7 crustaceans showing more or less of the exoskeleton and hypodermis dissected away to reveal the tear-drop-shaped sinus gland. A, *Carcinus*; B, *Crabo*; C, *Pagurus*; D, *Uca*; E, *Callinectes*; F, *Palaeomonetes*; G, *Libinia*.

tissue. It is slightly more easily observed in *Palaemonetes* than in *Crago*. In the remaining crustaceans mentioned above, it is not observed until the rather opaque dorsal exoskeleton, together with the pigmented hypodermis, has been removed from the stalk. The gland then stands out very conspicuously as a whitish or bluish-white tissue lying upon the comparatively transparent nervous tissue. It is a simple matter to remove the gland in one piece with watchmaker's forceps under the lower magnifications of a dissecting microscope. Figure 1, *A, B, C, D, E, F*, and *G*, shows roughly the relative sizes, shapes, and the positions of the glands as they appear in the different crustaceans which have been investigated in this regard. No special attempt was made to draw the different stalks to the same scale. All are left stalks.

In most of the injection experiments to be described the glands were dissected out of freshly excised eyestalks, placed in clean depression slides, allowed to dry, and then thoroughly ground with a glass rod. The ground mass was then extracted with the desired quantity of sea water and injected. In implantation experiments the glands were carefully maintained intact in sea water and were, within 2 or 3 minutes after removal, implanted as wholes into the abdominal region of hosts without sinus glands. By means of a fine glass capillary attached to an aspirator it has also been possible to remove the sinus gland, without apparent further injury to the animal. The results of these latter experiments will form the basis of a future report.

The sinus gland is removed with such ease that there appears to be little need for future investigators to resort to the coarser technique of triturating whole eyestalks when they are dealing with principles of this gland from such crustaceans as are mentioned herein.

THE SIZE OF THE CRAYFISH SINUS GLAND

To make this determination *Cambarus virilis* were selected, their carapace length measured, and their volume determined by water displacement. An eyestalk was next removed and put into a physiological salt solution. The eyestalk tissue was freed from the inclosing exoskeleton, and the sinus gland dissected out.

TABLE 1

Expt. No.	Carapace Length (Mm.)	Total-Body Volume (Mm. ³)	Eyestalk-Tissue Volume (Mm. ³)	Sinus-Gland Volume (Mm. ³)	Sinus Gland (Percentage of Eyestalk-Tissue Volume)	2 Sinus Glands (Percentage of Total-Body Volume)
1.....	36	13,000	2.605	0.0250	0.95	0.00039
2.....	36	11,500	2.932	0.0556	1.89	0.00096
3.....	31.2	7,500	2.000	0.0306	1.53	0.00082
4.....	25.5	4,800	1.446	0.0193	1.33	0.00080

The volumes of the sinus gland and remaining stalk tissue were then measured as follows. Each of the pieces of tissue was crushed between two glass slides, which were held apart by fragments of cover slip of known thickness. The outline was traced upon graph paper by means of a camera lucida, and the area calculated. Knowing the thickness of the preparations, the volumes were readily ascertained and expressed in cubic millimeters, percentage of total eyestalk tissue volume, or percentage of total-body volume. The results are given in Table 1.

SINUS GLAND EXTRACT VERSUS WHOLE-EYESTALK EXTRACT

After a few initial experiments had indicated that extracts of the isolated sinus gland had a tremendous chromatophorotropic effect upon *Uca* black chromatophores and *Palaemonetes* red ones, a series of experiments was designed to determine to what extent the chromatophorotropic influence of eyestalk extracts could be attributed to activity of the sinus gland. Sea-water extracts of sinus glands of different crustaceans were made, and each was injected into eyestalkless *Uca* with concentrated black pigment (having a black chromatophore index of one) and eyestalkless *Palaemonetes* with broadly dis-

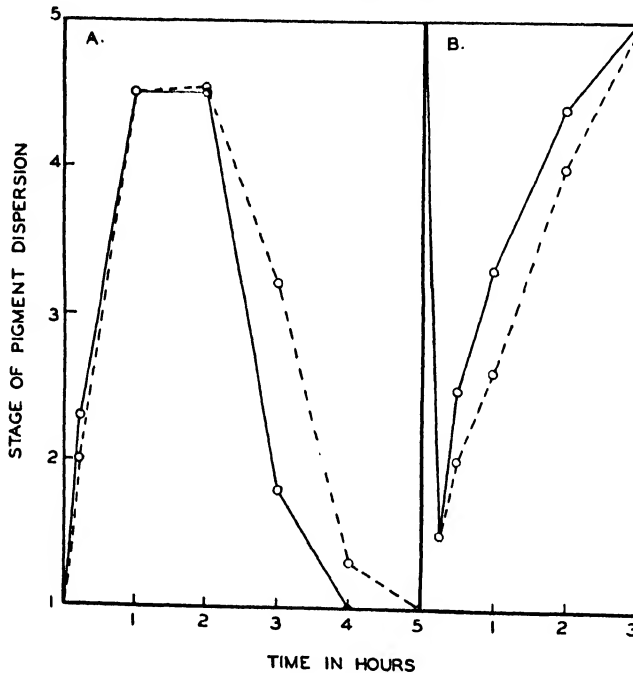


FIG. 2. Change in the average stage of pigment dispersion of the black pigment of *Uca* (A) and the red pigment of *Palaemonetes* (B), following injection of *Uca* sinus-gland extract (solid line) and *Uca* total-eyestalk extract (broken line), both extracted in the same quantity of sea water.

persed red pigment (having a red chromatophore index of five). In each case the same extract was injected into 10 *Uca* and 10 *Palaemonetes*. The dose given was always 0.05 cc. into *Uca* (at base of fourth walking leg) and 0.025 cc. into *Palaemonetes* (dorsal abdominal musculature). The concentration in the instance of *Uca* sinus gland was 1 sinus gland to 1 cc. of sea water, whereas in the other instances the concentration was 4 sinus glands to 1 cc. of solution.

The effects of injection of the extracts were measured quantitatively for the two types of chromatophores, using the change in the average chromatophore index with time as the criterion. For the red chromatophores of *Palaemonetes* their state was judged at 15, 30, 60, 120, and 180 minutes after extract injection. With *Uca* black chromatophores their state was judged at 15, 60, 120, 180, 240, and 300 minutes after injection.

The chromatophore-index method was essentially that employed by Hogben and his school (see Hewer, 1923; Hogben and Slome, 1931). A fully dispersed pigment mass was designated as 5, and a fully concentrated mass as 1. Different intermediate degrees of dispersion were called 2, 3, and 4, depending upon the degree. As an indication of the effect of an extract upon *Uca* black chromatophores, the sum of the average indices for each time interval was obtained, and the difference between it and 6 (no effect) was found. For the effect upon *Palaemonetes* red pigment the difference between 25 (no effect) and the sum of the average indices for each time interval was found. Such an index of the

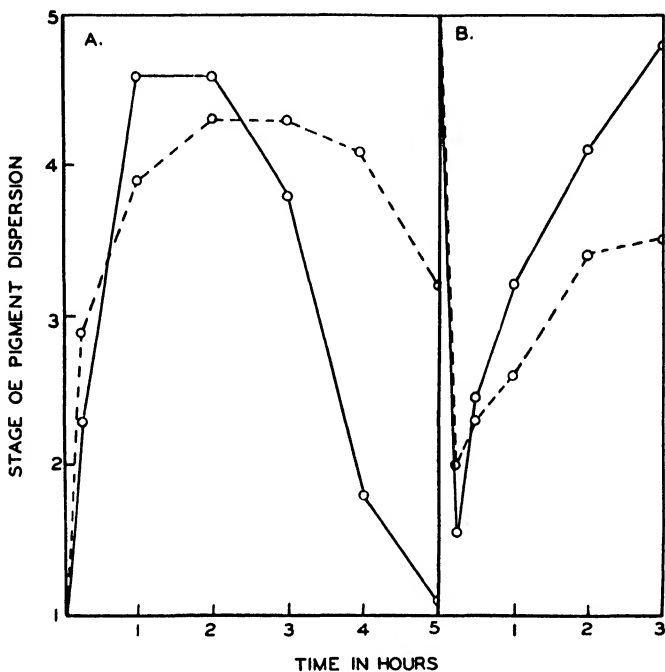


FIG. 3.—Change in the average stage of pigment dispersion of the black pigment of *Uca* (A) and the red pigment of *Palaemonetes* (B), following injection of *Carcinus* sinus-gland extract (solid line) and *Carcinus* total-eyestalk extract (broken line), both extracted in the same quantity of sea water.

total effect of an extract (the index value in the following descriptions) was believed reasonable, since it took into consideration both the magnitude of the chromatophore change and the duration of the effect.

The effects that were obtained with sinus-gland extracts were then compared with effects of whole-eyestalk extracts in which the extracts were prepared in the same way as for sinus glands, except that whole eyestalks were substituted for sinus glands. In so far as was possible, animals of similar sizes and weights were used with both sinus-gland and eyestalk experiments. Owing to lack of time, usually only 5 animals were injected. The experiments with each source of sinus gland and eyestalk will be discussed in turn.

Uca.—Four sinus glands were removed from 2 *Uca* weighing a total of 3.40 gm.,

and extracted in 4 cc. of sea water. This extract was injected into 10 *Uca* (0.05 cc. to each), having an average weight of 1.86 gm. Four eyestalks were removed from 2 *Uca*, weighing a total of 3.35 gm., and an extract of them injected into 10 *Uca* which had an average weight of 1.69 gm.

The same extracts were also injected into 2 lots of large female *Palaemonetes*, 7 animals to each lot. The *Palaemonetes* were roughly all the same size, though their weights were not determined.

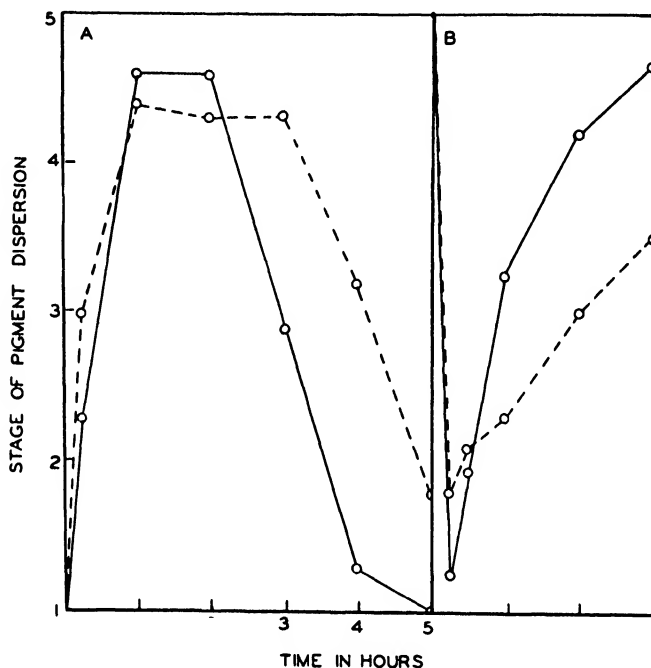


FIG. 4. Change in the average stage of pigment dispersion of the black pigment of *Uca* (A) and the red pigment of *Palaemonetes* (B), following injection of *Pagurus* sinus-gland extract (solid line) and *Pagurus* total-eyestalk extract (broken line), both extracted in the same quantity of sea water.

The results of these experiments are graphically shown in Figure 2. It is seen that the index of effect of sinus gland upon *Uca* was 9.1 and of whole eyestalk was 10.5. Thus the effect of the sinus gland by itself was 86.7 per cent of that of the whole stalk. Similarly, on *Palaemonetes* the sinus-gland-effect value was 7.7 as against 9.2 for the whole stalk. Here the sinus gland showed 83.7 per cent of the effect of the whole stalk.

Carcinus.—The average weight of the animals from which the sinus glands were removed was 56.4 gm. The average weight of the donors of the whole stalks was 56.0 gm. Each was made into an extract of 4 glands or stalks to 1 cc. of sea water. The effects of these extracts when injected into eyestalkless *Uca* and *Palaemonetes* are seen in Figure 3. On *Uca* the index value (I.V.) for sinus gland was 12.2 and for eyestalk 16.7. Here the sinus gland showed 73 per cent activity of the whole stalk. For *Palaemonetes*

red pigment the index value of sinus-gland activity was 8.9 and for eyestalk 11.2. Here the sinus gland showed 79.5 per cent of the activity of the whole stalk.

Pagurus.—Four sinus glands from animals with an average weight of 9.6 gm. and 4 eyestalks from animals of average weight of 10.1 gm. were extracted in concentration of 4 glands or stalks to 1 cc. sea water. The I.V. of the effect of the sinus gland on *Uca* was 10.6 and of whole eyestalk 15.0. Sinus gland thus showed 70.6 per cent activity of whole stalk. With *Palaemonetes*, I.V. of sinus gland was 9.7 and of eyestalk 12.3, with

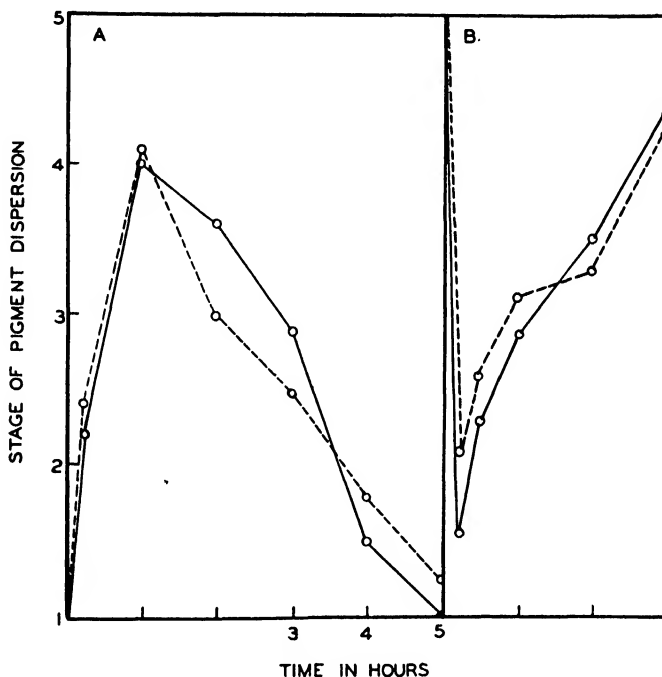


FIG. 5.—Change in the average stage of pigment dispersion of the black pigment of *Uca* (A) and the red pigment of *Palaemonetes* (B), following injection of *Palaemonetes* sinus-gland extract (solid line) and *Palaemonetes* total-eyestalk extract (broken line), both extracted in the same quantity of sea water.

sinus gland showing 79 per cent of total stalk activity. The results are plotted in Figure 4.

Palaemonetes.—The *Palaemonetes* from which the stalks and glands were removed weighed about 1 gm. apiece. The extracts of whole eyestalks and sinus glands alone were made in the usual concentrations. The effects were I.V. of eyestalk on *Uca*, 9.0; sinus gland on *Uca*, 9.2. Here the sinus gland showed even more effect than the whole stalk. This result is undoubtedly within the error of the method. On *Palaemonetes* red pigment I.V. of eyestalk was 9.7 and of sinus gland 10.4. Here again the sinus gland was more effective than the whole stalks. The results are shown in Figure 5.

Libinia.—Four sinus glands were removed from *Libinia* of average weight of 73.3 gm., and 4 eyestalks from *Libinia* of average weight of 83.5 gm. Extracts were made using

4 glands or stalks to 1 cc. of sea water. On *Uca* black pigment the I.V. of effect of eyestalk was 18.4 and of sinus gland was 10.7. Sinus gland thus showed 58.2 per cent of activity of whole stalks. On *Palaemonetes* red pigment the I.V. for eyestalk was 13.6 and of sinus gland 11.3. Sinus gland thus showed 83.2 per cent of total activity of the whole stalk. These results are seen graphically in Figure 6.

Callinectes.—Four sinus glands were removed from *Callinectes* of average weight of 87.5 gm. and 4 eyestalks from animals of average weight of 124 gm. These were ex-

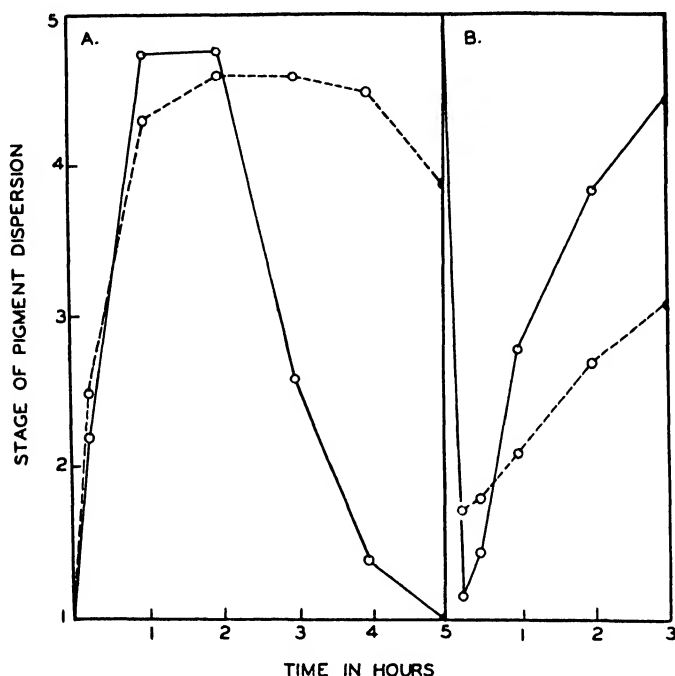


FIG. 6. — Change in the average stage of pigment dispersion of the black pigment of *Uca* (A) and the red pigment of *Palaemonetes* (B), following injection of *Libinia* sinus-gland extract (solid line) and *Libinia* total-eyestalk extract (broken line), both extracted in the same quantity of sea water.

tracted in sea water in concentration of 4 stalks or glands to 1 cc. The I.V. of effects on *Uca* black were eyestalk, 16.4; sinus gland, 12.3. Thus sinus glands showed 75 per cent of total stalk activity. On *Palaemonetes* red pigment the I.V. of effects were eyestalk, 14.0; sinus gland, 13.2. Here sinus gland showed 94.2 per cent of total stalk activity. Figure 7 shows the results of this experiment.

Crago.—Four sinus glands and 4 eyestalks were removed from *Crago* weighing between 0.8 and 1.0 gm. each. These were extracted in sea water in the concentration of 2 glands² or stalks to 0.5 cc. The I.V. of effect on *Uca* black pigment was eyestalk, 13.7; sinus gland, 10.7. Sinus gland showed 78.1 per cent of total stalk activity. Strangely

² Because of the difficulty of removal of this organ in *Crago*, there is some question of the exact concentration of the extract. At any rate, it lies between the concentration indicated and a value 50 per cent greater.

enough, these same two extracts when injected into *Palaemonetes* showed I.V. effect of eyestalk as only 0.2 while the sinus gland showed 4.0. These results are seen in Figure 8.

General.—Taking all the results together but excluding the response of *Crago* tissues upon *Palaemonetes*, the value of which for some reason falls completely out of line with the remainder, the average percentage of total chromatophorotropic activity of the eyestalk to be found in the minute sinus gland by itself is 82.5 per cent. This leaves a

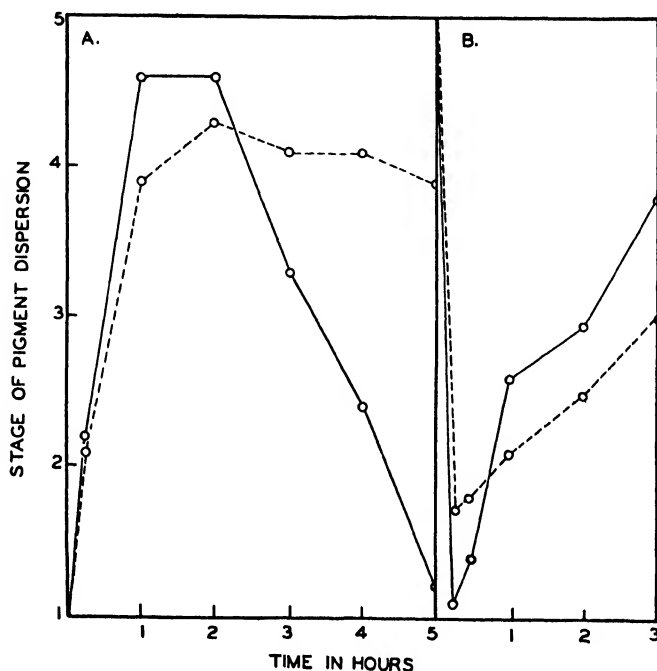


FIG. 7.—Change in the average stage of pigment dispersion of the black pigment of *Uca* (A) and the red pigment of *Palaemonetes* (B), following injection of *Callinectes* sinus-gland extract (solid line) and *Callinectes* total-eyestalk extract (broken line), both extracted in the same quantity of sea water.

residue of activity of the stalk of 17.5 per cent. There seems to be no reason to suspect that the difference of 17.5 per cent need be accounted for by anything other than the normal diffusion of materials from the gland to other eyestalk tissues during the course of gland activity. It is also unlikely that the gland is removed without any loss of some of its constituent cells or cell contents.

SINUS-GLAND EXTRACTS VERSUS EXTRACTS OF STALK REMAINDER

General confirmation of the preceding results was obtained by comparing the effects of sinus-gland extracts of *Carcinus* with effects of extracts of the remaining stalk tissue extracted in the same quantity of sea water. The only test employed in this experiment was the red pigment of eyestalkless *Palaemonetes*.

The extracts were made up as absolute ethyl alcohol extracts of dried tissue according to the method of Abramowitz (1936). The ultimate concentrations used were 1 sinus-glandless stalk or 1 sinus gland to 0.1 cc., and these same extracts diluted ten times. The dose injected into the test animals (large eyestalkless female *Palaemonetes*) was always 0.02 cc., and 5 animals were injected to determine the I.V. of the particular extract.

The results are seen in Figure 9. It is readily calculated that at the stronger concentration the I.V. of effect of the sinus gland is 12.4 and the glandless stalk 7.3. Thus, of

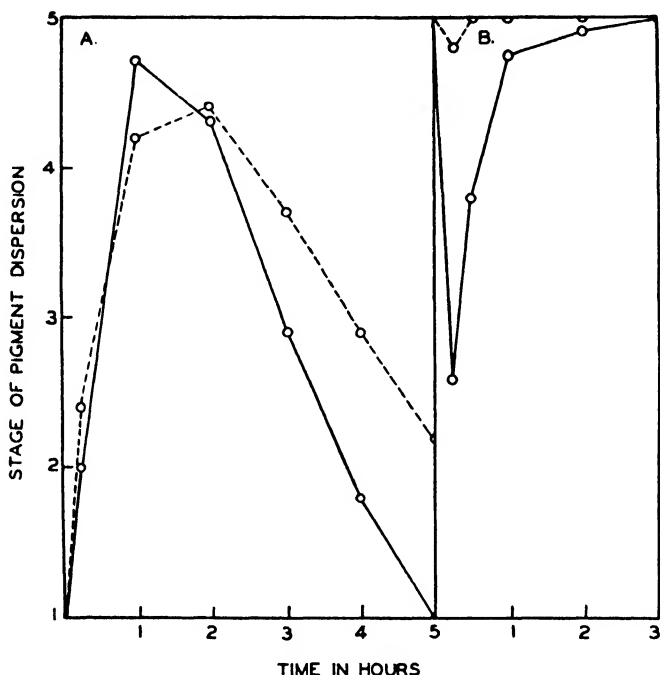


FIG. 8.—Change in the average stage of pigment dispersion of the black pigment of *Uca* (A) and the red pigment of *Palaemonetes* (B), following injection of *Crango* sinus-gland extract (solid line) and *Crango* total-eyestalk extract (broken line), both extracted in the same quantity of sea water.

the whole stalk, the sinus gland is showing 63 per cent of the total effect, and the remainder of the stalk tissue 37 per cent. At the greater dilution these proportions are respectively 97 per cent and 3 per cent. The average of these two values indicates that 80 per cent of the total stalk chromatophorotropic effect was in the sinus gland proper and that the remainder of the stalk tissue showed 20 per cent. This is in remarkable agreement with the average results of the earlier experiments performed in a slightly different manner that gave the values 82.5 per cent and 17.5 per cent and more particularly in agreement with the results of *Carcinus* extracts on *Palaemonetes* which gave the values 79.5 per cent and 20.5 per cent. The apparent exact repeatability of the

Carcinus results can scarcely be attributed to more than chance coincidence, considering the type of experiment.

CHROMATOPHOROTROPIC ACTIVITY OF SINUS-GLAND IMPLANTS

Sixty large female *Palaemonetes* were deprived of their eyestalks. The red pigment took up its usual state of complete dispersion. At the end of 24 hours the animals were divided into two lots of 30 each. Into the ventral region of the last abdominal segment of each animal of lot *A* was implanted a whole *Carcinus* sinus gland by means of a fine

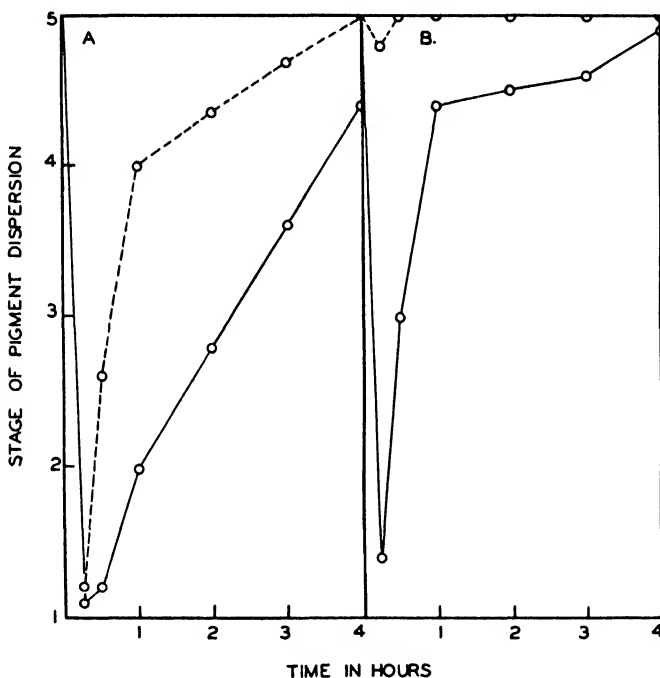


FIG. 9.—Comparative effects of injection of extracts of sinus gland (solid line), and of sinus-glandless eyestalk (broken line) upon the red pigment of *Palaemonetes* as it appears when using 1 glandless stalk or 1 gland to 0.1 cc. of sea water (*A*) and 1 glandless stalk or 1 gland to 1 cc. sea water (*B*).

glass pipette which was inserted at the base of a uropod. Each animal of lot *B* received a similar puncture and an equivalent dosage of sea water, but no gland. Each of the animals of lot *A* showed the sinus-gland implant clearly visible through the transparent exoskeleton and became lighter and bluish almost immediately as if they had received an injection of extract of the gland. Lot *B*, the controls, showed no chromatophoric response. At the end of 1, 3, 5, and 10 days, the average I.V. of the state of red-pigment dispersion of each lot was determined. These are plotted in Figure 10. The results indicate that the effect of the implant is to concentrate the red pigment and that the effect is maximum during the first day or two and then gradually wears off during 6 or 7 days. Interestingly enough, this is just about the extent of lengthening of the life of

sinus-glandless animals by sinus-gland implants (Brown, 1938; Brown and Cunningham, 1939). In individual animals there was an interesting correlation observed between the time of loss of chromatophoric influence and the time the gland ceased to maintain its clear bluish-white appearance. Those glands which came to lie directly alongside the ventral nerve cord were those that maintained their activity longest.

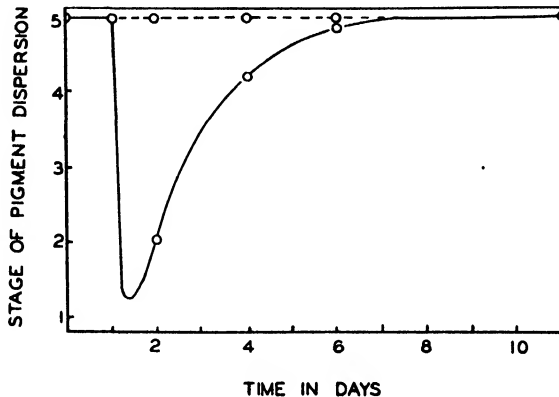


FIG. 10.—The average stage of dispersion of red pigment in two lots of 30 eyestalkless *Palaemonetes*, into one lot of which *Carcinus* sinus glands were implanted into the abdomen after 1 day (solid line), the other lot receiving a comparable puncture but no implant (broken line.)

The white chromatophores showed no significant difference in the two lots, the average white chromatophore I.V. of the two lots at 1, 3, and 5 days after implantation was, with implant, 1.9; and without it, 2.0; with no consistent change during those 5 days.

CONCLUSIONS

A definite glandular body, the sinus gland, has been located in the fresh eyestalk of seven crustaceans, *Callinectes*, *Libinia*, *Carcinus*, *Pagurus*, *Crago*, *Palaemonetes*, and *Uca*. From all of these forms the gland is easily removed by dissection, and in two of them, *Crago* and *Palaemonetes*, it can be clearly seen in the normal living animal under suitable lighting. The gland appears to be the sole tissue of the eyestalk which secretes a significant quantity of chromatophorotropic material influencing the red pigment cells of *Palaemonetes* and the black ones of *Uca*. Although the gland constitutes only about a hundredth part of the total-stalk tissue, its effects have been shown to be 80 per cent or more of that of the whole stalk, and there is no justification for belief that the remaining 20 per cent of the activity originates from any other tissue but rather results from normal and artificially induced escape of material from the gland proper. These results have been obtained from three different types of experiments: (1) quantitative comparison of effects of sinus-gland extracts with those of the whole-eyestalk extract; (2) quantitative comparison of effects of sinus gland by itself with effects of eyestalk tissue from which only the sinus gland had been removed; and, finally, (3) implantation of sinus glands into the ventral abdominal region of animals having their sinus glands removed.

All the crustaceans examined have the same type of gland, judging from its gross

appearance and from its location. All the sinus glands have principles effective on *Uca* black pigment and on *Palaemonetes* red pigment. Furthermore, for each species the relative effects of its sinus gland as compared with its whole eyestalk upon the *Uca* black or *Palaemonetes* red pigment have been generally the same. Thus, at least in the majority of crustaceans, the whole eyestalk and sinus gland have qualitatively the same effect according to the criterion used. This further eliminates the possibility that another eyestalk gland is affecting either of these chromatophore types, unless it functions in the same manner as the sinus gland.

Summarizing briefly, the evidence indicates that the source of the chromatophoretropic substance of the crustacean eyestalk is the sinus gland. As far as the *Uca* black chromatophores and the *Palaemonetes* red ones are concerned, this gland will account wholly for the activity of the eyestalk extracts.

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THE EFFECT OF CONCENTRATION OF COLPODA DUODENARIA ON THE TIME REQUIRED FOR ENCYSTMENT IN FOOD-FREE MEDIUM

(Four figures)

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THE resting cysts of *Colpoda duodenaria* offer promising material for experimental studies, both qualitative and quantitative, on protoplasm in a relatively dedifferentiated state. In order to be certain that the material prepared at different times can be considered as physiologically the same, it is necessary to know which environmental factors must be controlled during the growth of the cultures and the preparation of the cysts.

It has been shown (Taylor and Strickland, 1938, 1939) that

1. The formation of resting cysts in *C. duodenaria* follows the reduction in amount of the available food supply in the medium.
2. Those organisms which do not form resting cysts in the absence of food will form "rhythmic" cysts, i.e., the *Colpoda* will encyst but will spontaneously reorganize and excyst, and this process will be repeated until, owing to depletion of the body substance, death ensues.
3. The percentage of resting cysts formed varies directly with the logarithm of the concentration of the protozoa.
4. The percentage of resting cysts formed at constant concentration of organisms decreases as the volume of medium decreases between $\frac{1}{4}$ and $1/40,000$ cc. It seems that the protozoa condition the medium, promoting thereby the change from cyst to resting cyst. It is of importance to know at what stage of the process of encystment this interaction among the *Colpoda* begins to affect them. This paper shows the effect of the concentration of the organisms on the duration of the precyst period of *Colpoda* in various volumes of food-free medium.

MATERIAL AND METHODS

The protozoa, food, culture medium, and much of the technique were as described elsewhere (Taylor and Strickland, 1938, 1939). About one million resting cysts in a dish were washed to remove unattached debris and then induced to excyst by the addition of yeast extract. As soon as the first free-swimming *Colpoda* were seen, the yeast extract was poured off, and balanced medium added. Forty-five minutes later, when excystment was almost complete, the culture was decanted into a clean dish and fed with that amount of a suspension of bacteria determined by experience, which will allow the organisms to grow large enough to form quadrigenic division cysts.

The resulting culture of approximately four million *Colpoda* was washed twice in the centrifuge to remove cyst membranes and most of the bacteria. About fifty thousand of these washed protozoa in $\frac{1}{4}$ cc. of medium were put into each of a number of Colum-

bia dishes. They formed resting cysts, which adhered so firmly to the clean glass surface that they could be thoroughly washed as often as required with sterile balanced medium. The cysts in these Columbia dishes provided uniform material for later experiments.

A rigid time schedule was followed. For each series of tests the cysts in one of the Columbia dishes were thoroughly washed before yeast extract was added. After a given period the yeast extract and the first few excysted protozoa were poured off and replaced by sterile balanced medium. After a fixed interval the excysted *Colpoda* were washed in 100 cc. of sterile balanced medium in the centrifuge to remove the cyst membranes. The washing was repeated, and the *Colpoda* thus obtained were sufficiently clean to be regarded as food free.

The end of the above washing procedure was arbitrarily chosen as time "zero," from which to reckon the duration of the precyst period. The end of the precyst period was arbitrarily determined as the moment when the encysting organism, having withdrawn the cilia through the newly secreted ectocyst, began to rotate within the membrane. This, of course, is not the end of the encystment process.

Micropipettes were calibrated to $1/40$, $1/200$, $1/1,000$, and $1/5,000$ cc. To obtain drops of $1/25,000$ cc., it was found possible to use a $1/5,000$ -cc., pipette and to distribute its contents in five apparently equal drops with sufficient speed and accuracy. For drops of $1/40$ and $1/200$ cc. Petri dishes with closely fitting covers were used as observation chambers. For smaller drops, cover slips reversed onto hollow-ground slides were used. For all cases a saturated atmosphere was maintained by additional drops of distilled water.

Five thousand organisms in a drop proved to be the greatest number whose encystment could be followed with sufficient quantitative accuracy. The required number of *Colpoda* was injected into the drops of $1/40$ and $1/200$ cc. by means of injection pipettes of extremely small caliber. In order not to increase appreciably the volume of the drop when 5,000 organisms were injected into it, the washed *Colpoda* were momentarily concentrated to many millions per cubic centimeter before they were picked up by the injection pipette.

For drops of $1/1,000$ cc. and smaller, the food-free *Colpoda* were diluted to approximately the concentration desired, and the requisite number of the protozoa were then picked up with the calibrated pipettes.

These small drops were placed on cover slips, which were immediately reversed onto hollow-ground slides, so that decrease in volume by evaporation was not appreciable. During the distribution of the smaller drops the relative humidity in the constant-temperature room (20° C.) was around 90 per cent.

EXPERIMENTAL

With the material and by the methods described in the previous section, the encystment was observed of concentrations of *Colpoda* between 1,000 per cubic centimeter (25 per drop) and 200,000 per cubic centimeter (5,000 per drop) in drops of $1/40$ cc. Cysts were always noted first in the drops with the highest concentration, but the drops with lower concentrations followed closely with one or more cysts. This is of interest because it shows that at all concentrations encystment begins approximately at the same time.

From five to seven counts of the cysts in each drop of the series were made during encystment. The mean of the times at the beginning and end of the counting period was noted as the time of the observation; fractions of a minute were not noted. An example of such a series is given in Table 1 and is plotted on linear paper in Figure 1;

TABLE 1
SERIES D.78: ENCYSTMENT OF DIFFERENT CONCENTRATIONS IN DROPS OF 1/40 CC
(Time in Minutes from Zero)

No.	Time	Cysts	Percent- age	Time	Cysts	Percent- age	Time	Cysts	Percent- age
1...	100	100	2 0	122	573	11.4	140	2,000	40.0
2...	103	20	2 0	128	120	12.0	144	252	25.2
3...	105	2	0.7	131	19	7.0	150	32	11.9
4...	136	1.5	4 8	155	2.5	8.1	175	4	12.9
1...	163	3,500	70.0	180	4,400	88.0	205	4,900	88.0
2...	165	500	50.0	183	750	75.0	206	900	90.0
3...	170	82	30.4	190	153	56.7	212	198	73.4
4...	198	7.5	24.2	218	13	42.0	260	19	61.3

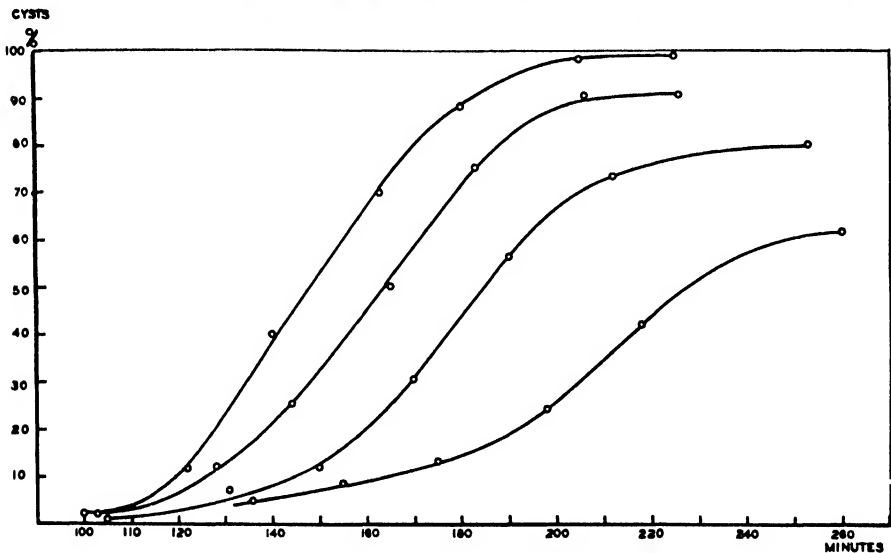


FIG. 1. Series D. 78. Encystment curves at different concentrations of *Colpoda*. Volume of the drop: 1/40 cc. Ordinates: percentage of cysts at time of observation; abscissae: time of observation in minutes from zero.

the ordinates are the number of cysts per cent of the number of organisms in the drop; the abscissae are the times of observations in minutes from zero.

Encystment evidently proceeds more rapidly as the concentration of organisms increases. In those cases in which the concentration of *Colpoda* is not sufficient to induce

all the protozoa to form resting cysts (Taylor and Strickland, 1939) the curves may never reach the 100 per cent line, because the first animals to encyst may be ready to emerge before all the organisms have encysted. The upper ends of the curves then deviate from the sigmoid form. But, if we take as a criterion of the effect of the concentration

TABLE 2
 t_{50} FOR CONCENTRATIONS OF SERIES D.78

No.	<i>Colpoda</i> per Drop	<i>Colpoda</i> per Cubic Centimeter	t_{50}
1.	5,000	200,000	150
2.	1,000	40,000	164
3.	270	10,800	185
4.	31	1,240	230

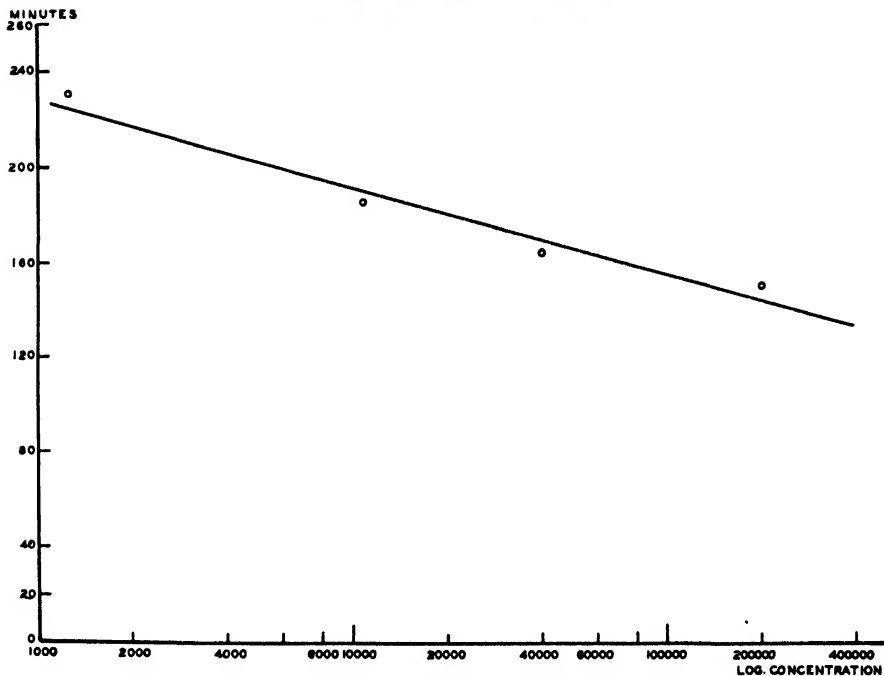


FIG. 2.—Series D. 78. t_{50} at different concentrations of *Colpoda*. Volume of the drop: 1/40 cc. Ordinates: t_{50} in minutes from zero; abscissae: logarithm of the concentration of *Colpoda*.

of protozoa on the rapidity of encystment the time required for the encystment of 50 per cent of them, the curves above that point are not so important.

If these times, t_{50} , are taken (Table 2) from Figure 1 and plotted (Fig. 2) against the logarithm of the concentration, it appears that the time required for 50 per cent of the *C. duodenaria* to form cysts in a drop of a given volume is an exponential function of the

TABLE 3
MEAN VALUES OF t_{50} AT DIFFERENT CONCENTRATIONS
IN DROPS OF $1/40$ CC.

Group	Cases	Concentration	t_{50}	Standard Deviation	Standard Error
1.....	11	1,460	201	33.3	10.0
2.....	14	9,880	174	26.4	7.1
3.....	8	45,650	156	21.6	7.6
4.....	10	198,000	151	15.0	4.7

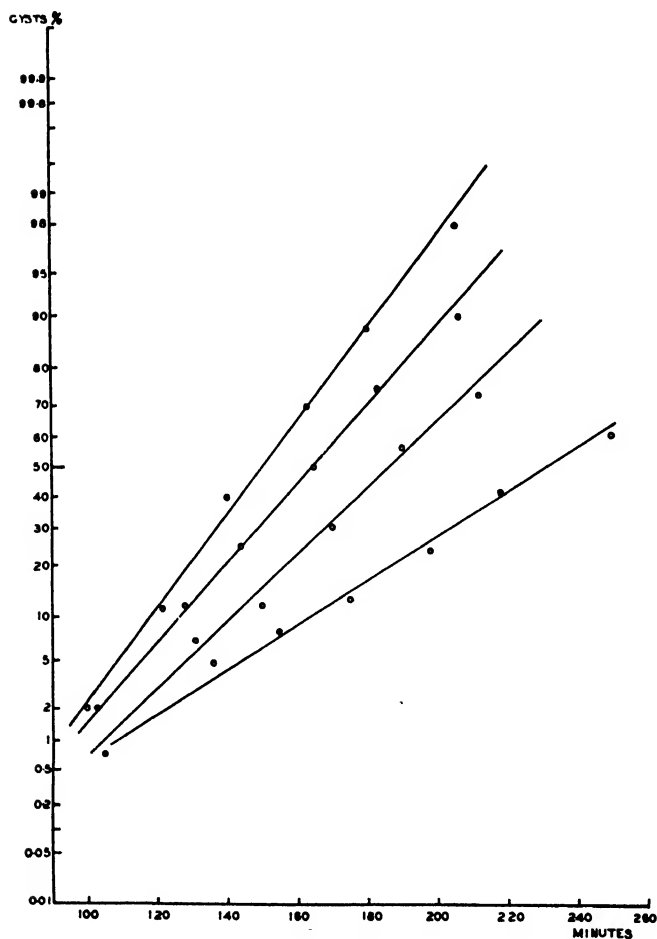


FIG. 3.—Series D. 78. Encystment curves at different concentrations of *Colpoda*. Volume of the drop: $1/40$ cc. Ordinates: percentage of cysts (probability scale); abscissae: time of observation (arithmetic scale).

concentration of the protozoa. Thus, for the series shown in Figure 1 the time required for encystment of 50 per cent of the *Colpoda* in 1/40 cc. of food-free medium decreases by about 11 minutes when any concentration is doubled.

At every repetition of the experiment this logarithmic relationship was substantiated, but as the curves for each series did not exactly coincide, probably significant of some as yet imperfectly controlled factors in the technique or environment. eight final series were tested. As nearly as possible the same concentrations were studied in each series, so that the data for the 43 cases could be assembled in four groups.

TABLE 4
ASSEMBLY INTO CONCENTRATION GROUPS OF THE STANDARD
DEVIATIONS OF t_{50} FOR ALL VOLUMES OF THE DROP

Group	Concentration	Standard Deviations	Mean Standard Deviation M	Standard Deviation of M
I	1,000-6,000	33.3 24.1 19.3	25.6	5.82
II.....	6,000-50,000	26.4 26.4 19.6 20.2 21.6	24.0	2.32
III.....	50,000-300,000	15.0 12.7 19.9 14.4 17.2	15.8	2.49
IV.....	300,000-2,000,000	17.5 16.2 12.0 15.4 10.8	14.4	2.55
V.....	2,000,000-10,000,000	10.9 10.5	10.7	0.2
VI.....	Above 10,000,000	2.6	2.6

Table 3 gives the data for the four groups in 1/40 cc., and Figure 4 shows the straight line through the mean value of t_{50} at the mean concentration for each group.

In some of these cases, especially in those containing few *Colpoda*, it was difficult to determine on linear paper the correct position of the sigmoid curves and so of t_{50} . The data were therefore plotted on arithmetic-probability paper (Hazen, 1914; Whipple, 1916), which transforms the curves resulting from random distribution or variation of physiological factors into straight lines (Fig. 3). These lines are more easily determined than the sigmoid curves, though it may be necessary to ignore data above 65 per cent if excystment has begun.

Eight similar series with 55 cases were made in drops of $1/200$ cc. Results similar to those for $1/40$ cc. were obtained. The range of concentrations is between 1,600 and 1,000,000 *Colpoda* per cubic centimeter (5,000 per drop). The straight line through the mean values of t_{50} at the mean concentration for each of the four groups is shown on semilogarithmic paper in Figure 4. It is nearly, but not quite, the same as for $1/40$ cc.

For drops of $1/1,000$ cc. there are 145 cases in four groups. In the three smallest sizes of drops each organism represents a considerable concentration per cubic centimeter. In the lower concentrations in these small drops there are not enough organisms to determine adequate encystment curves, so that in each series 5-10 cases containing

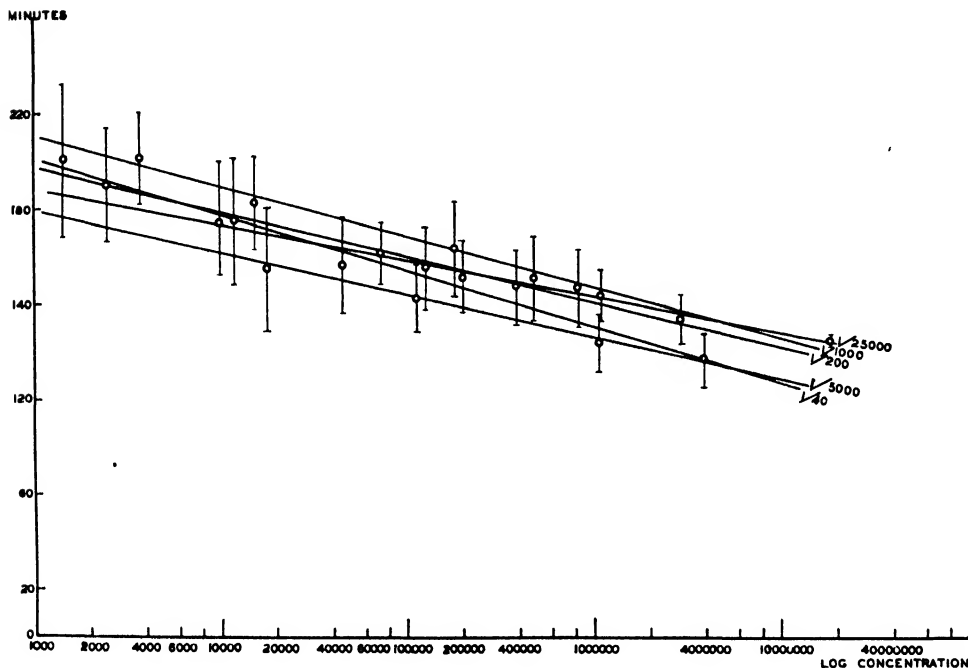


FIG. 4.--Synopsis of mean t_{50} curves for the five different volumes of the drop, with the standard deviations of t_{50} from the mean value of t_{50} for each of the twenty groups. Ordinates: t_{50} in minutes from zero; abscissae: logarithm of the concentration.

the same low concentration of *Colpoda* were observed; these cases were then combined in a subgroup to give adequate data for a reasonably accurate curve. From these subgroups values of t_{50} were obtained, and they, weighted with the number of cases in their subgroups, were used to determine the mean value of t_{50} for the low-concentration group.

Similar experiments were made in drops of $1/5,000$ cc. (seven series with 84 cases in four groups), and in drops of $1/25,000$ cc. (four series with 100 cases in five groups). The mean curves showing the relationship between t_{50} and the concentration of the protozoa are also shown in Figure 4; they are near, but do not coincide with, those for the other sizes of drop. The standard deviation of the mean of each group was calculated for all five sizes of drop and is represented in Figure 4 by a vertical line extending to equal distances above and below each point.

If the points in Figure 4 are divided into six groups of concentrations and if the mean M of their mean deviations is determined for each group, it will be seen (Table 4) that (1) these standard deviations are of approximately the same order of magnitude within each group, i.e., the standard deviations of M are small; and (2) M increases as the concentration decreases. Since the standard deviations of the points within each group (Fig. 4) all overlap each other and are of about the same magnitude within each group, it must be supposed that under ideal conditions of technique and environment the five curves would coincide. This means that the time required for 50 per cent of the organisms to encyst in food-free medium is independent, within the range here considered, of the volume of the drop containing them.

DISCUSSION

Colpoda duodenaria, regardless of its size, will, in food-free medium, proceed to form a cyst after a certain delay. Part of this delay may be due to the presence of undigested food, which is therefore equivalent to food in the external environment. It is as yet undecided, whether this ciliate stores any such substances as oils or glycogen. This seems improbable, because such reserves should act as inhibitors of encystment to prolong the pre-encystment delay in food-free medium. Preliminary experiments indicate that this delay in a given concentration of *Colpoda* is remarkably constant, regardless of the size and previous feeding of the organism.

Taylor and Strickland (1939) have shown that certain environmental conditions, namely, the concentration of the organisms during encystment and also the volume of the drop of food-free medium in which they are contained, determine the percentage of the cysts that will become resting cysts. It is important to know if these same conditions also influence the precyst delay and the dedifferentiation period of all the organisms, regardless of their subsequent fate. If so, then resting cysts might be physiologically different according to the concentration of the organisms and perhaps the volume of medium in which they were formed.

The selected criterion of the influence of these factors on the precyst period, the time t_{50} between the end of the washing procedure, arbitrarily chosen as zero, and the moment when the encysting protozoön begins to rotate within the ectocyst, can be determined with sufficient accuracy on arithmetic-probability paper. Theoretically, the encystment curve of strictly uniform organisms is a vertical line. The probability scale, representing the normal law of error, tends to eliminate the effect on the sequence of observations of the random distribution of uncontrollable differences in the physiological condition of the *Colpoda* at the beginning of the experiments.

Throughout the process of evolving a satisfactory technique the results obtained, showing the relationship between the concentration of *Colpoda* and the time required for 50 per cent of them to become cysts, were qualitatively the same, though quantitatively somewhat variable. The straight lines on semilogarithmic paper were always straight lines. Some were parallel but shifted by a few minutes on the axis of the ordinates; it seems quite probable that this was due in part to the indeterminate position of zero.

However strictly the time schedule be adhered to, there may be certain unspecifiable factors, temporary promoters or inhibitors of encystment, that might induce a variation in the mean physiological condition of the organism in different experimental series. Such a factor might be the degree of success in separating the protozoa from the cyst

membranes with adhering bacteria in the first of the two final washings in the centrifuge. The fluctuation in the angle of the t_{50} curve on semilogarithmic paper with the axis of the abscissae is also probably due to an undetermined variation in technique. The steeper the line, the more powerful is the encystment-promoting action of an increase in the concentration of the organisms.

The encystment curves (Fig. 1) for different concentrations of *Colpoda* in drops of 1/40 cc. show that encystment is promoted by an increase in the concentration. In the lowest concentration (1,240 *Colpoda* per cubic centimeter) about 20 per cent of them can be expected to form resting cysts (Taylor and Strickland, 1939). Encystment is so slow that about two hours after the first cysts have appeared the other 80 per cent have begun to excyst, so that just after the point of 50 per cent encystment the curve begins to flatten. The three lower curves all show some excystment beginning at two hours after the first cysts were formed. The highest concentration (200,000 per cubic centimeter) should finally reach 100 per cent, and all should be resting cysts.

Figure 2 shows that the encystment time t_{50} decreases proportionately with the increase in the logarithm of the concentration. This indicates an encystment-promoting conditioning of the environment by the organisms. The eight series in drops of 1/40 cc. vary considerably among themselves. The standard deviations and the standard errors for each group are high; since 5,000 *Colpoda* per drop is the maximum number that can be observed with sufficient speed and accuracy, the concentrations are comparatively low.

Figure 4 and Table 4 show that, when the points in Figure 4 are divided into six groups of concentrations, the mean M of the standard deviations of the points in a group increases as the mean concentration of the group decreases. This shows that the encystment-promoting factor is more efficient in that it produces more uniform, as well as more potent, results in high concentrations of the protozoa.

If we take from the five curves in Figure 4 the values of t_{50} at any one point, e.g., 100,000 *Colpoda* per cubic centimeter, we find

153.5 minutes for drops of 1/40 cc.	
159.0	1/200
167.5	1/1,000
143.0	1/5,000
157.5	1/25,000

The mean is therefore 156 minutes ± 8 minutes, or 156 minutes ± 5.1 per cent. The standard deviation (8 minutes) is much smaller than the standard deviations of the five curves in this region (group III, Table 4), and these five standard deviations all overlap each other. There can be little doubt that under ideal conditions the five curves would coincide. The time required for 50 per cent of the *Colpoda* to encyst at any concentration of organisms is therefore independent of the volume of the drop within the range of concentrations and in the sizes of drop considered here. This indicates that the encystment-promoting factor is not identical with the factor inducing the change from cyst to resting cyst, whose potency varies with the volume of the drop.

If the curve illustrating the relationship between t_{50} and the concentration continues unchanged, it should be theoretically possible to concentrate the protozoa so highly that when placed in food-free medium and with no unassimilated food in the body they

would immediately begin the secretion of a cyst membrane. In such a case the t_{50} curve would approach the arbitrarily chosen zero line.

If the curve is extrapolated to a concentration of one *Colpoda* per cubic centimeter, the required t_{50} should be about 250 minutes. One *Colpoda* in a liter of medium should require about 320 minutes. It seems, therefore, possible that the encystment-promoting factor, produced in the organism as an indirect result of an insufficiency of food, may condition the internal environment so as to induce encystment. If some of this promoting agent were lost by diffusion into the medium, the greater the concentration of organisms, the less would be this loss by diffusion.

At constant concentration of *Colpoda*—i.e., the number of organisms per unit volume of medium—the concentration of the factor in the medium due to diffusion from the protozoa would be independent of the volume of medium if the factor is not affected by the surface-volume ratio of the drop. This is in marked contrast with the properties of the factor determining the percentage of resting cysts, which is apparently greatly affected by the surface-volume ratio.

If a threshold concentration of the factor in the external environment were necessary to induce encystment, one would not expect the curves in Figure 1 to start at the same time; the beginning of encystment should rather be delayed, depending on the number of organisms per cubic centimeter, until the factor had reached the requisite concentration; the encystment curves would then all be parallel and similar.

SUMMARY

This study of the time t_{50} required for 50 per cent of various concentrations of *C. duodenaria* to form cysts in various volumes of food-free medium, reveals, for concentrations between 1,000 and 20,000,000 *Colpoda* per cubic centimeter, that (1) the time t_{50} decreases proportionately to the increase in the logarithm of the concentration of organisms, (2) the time t_{50} is independent of the volume of the drop containing the protozoa. The possibility of an encystment-promoting factor, indirectly induced by an insufficiency of food, is briefly discussed.

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NOTICE

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THE PRIMARY DEVELOPMENT OF THE SKELETON IN NERVELESS AND POORLY INNERVATED LIMB TRANSPLANTS OF CHICK EMBRYOS¹

(Three figures and two plates)

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THE nervous system participates in several indirect ways as a factor in the development of the skeleton. It has a trophic influence on the developing limb bud, including its skeleton. This was shown for the frog, where the noninnervated but otherwise undisturbed hind limb exhibits a growth reduction of about 12 per cent (Hamburger, 1928). A similar effect, though less clearly analyzed, seems likely to exist in the case of the chick limbs. The most obvious indirect action of the nervous system would be by way of excitation of muscular activity. The question of whether functional movement of adjacent elements is a factor in molding and shaping the cartilages and bones and particularly the articulating surfaces and in preventing fusions of joints has been widely discussed and is still controversial. The experimental analysis of these problems has been ably reviewed by Murray and Selby (1930) and by Murray (1936). They arrive at the conclusion that, whereas the gross form of the individual skeletal elements is attained by self-differentiation from early stages on, such extrinsic factors as outlined above are essential in perfecting and refining the details and in forming functional joints.

These conclusions are based largely on experiments on the limb skeleton of the chick, using chorio-allantoic grafts and tissue-culture experiments as methods of analysis. Our method of transplanting limb primordia to the flank or into the coelomic cavity has certain advantages over the other methods. It provides for optimal conditions as far as nutrition and space requirements for the developing limb are concerned. Furthermore, very young stages of limb primordia may be transplanted, and the observations may be extended over a long period of time, possibly even beyond hatching. In an extensive series of such transplants (Hamburger, 1939) a number of normally shaped, nerveless, or poorly innervated limbs were obtained, the skeletons of which were studied in detail. The present investigation is limited to the differentiation of the cartilaginous skeleton and the early phases of osteogenesis.

¹ This investigation was aided by a grant of the Rockefeller Foundation. The authors are indebted to Miss Marie Jakus for the preparation of the drawings and to Mr. E. L. Keefe for the preparation of the photographs.

MATERIALS AND METHODS

Wing and leg primordia of chick embryos of 51–72 hours of incubation were transplanted heterotopically to hosts of the same stages. For details of technique and seriation of stages see Hamburger (1938, 1939). Forty-nine normal and 27 malformed cases were selected for the present investigation. The latter are included in order to study the type and range of skeletal abnormalities resulting from transplantation. Atypical short outgrowths which have been obtained mainly from late transplanted limb buds (stages 5 and 6) are omitted. The transplants are fixed at an age of 9–21 days, most at the age of 9–10 days.

Fourteen cases (5 wings, 9 legs) were sectioned, stained with iron hematoxylin, and their nervous and skeletal systems reconstructed. Fifty-four cases (29 wings, 25 legs) were stained *in toto* with methylene blue, following Lundvall's technique, which is a differential stain for cartilage.

The method used by us is briefly as follows: The whole embryos are stained in a solution of methylene blue in 70 per cent alcohol (0.25 gm. per 100 cc. of alcohol) for 48 hours, destained in acid alcohol for 2–3 hours, washed and run up through the alcohols (70 per cent–95 per cent–absolute), cleared in a solution of equal parts of absolute alcohol and benzene for 6 hours, and then in pure benzene for 12 hours. They are hardened and stored in a solution of three parts oil of wintergreen to one part benzyl benzoate, according to the Spalteholz method.

Two legs (21 days) were stained differentially for bone with alizarin red, and 6 transplants (2 wings, 4 legs; 18 days and older) were dissected.

THE INNERVATION OF THE TRANSPLANTS

The innervation of transplanted limbs has been described in detail (Hamburger, 1939). It is found that only transplants located near the spinal cord receive an appreciable amount of nerve supply, whereas transplants located ventrally or inside the coelomic cavity are very poorly innervated or nerveless. The quantitative development of all transplant nerves is surprisingly poor. Movements of transplants are observed occasionally but are never extensive. They are found only in transplants located near a host limb and supplied, at least in part, by limb nerves. Absence of visible motion, however, does not exclude the possibility that individual muscles may have been functional.

Of the present material, 4 wings and 5 legs are definitely nerveless, as ascertained in sections or by dissecting. These are located in the coelom or loosely attached to the mesenteries of the umbilical cord. They are, then, the "crucial" cases. The innervation of the cases stained in methylene blue and alizarin red was not observed directly. Most of them are located in the ventral body wall, in the coelom, or in the umbilical cord. Their innervation must be absent or, at best, very poor. Even though innervated, they would not be capable of extensive motility, because almost all transplant girdles are very poorly developed (see below) and are imbedded in the soft tissues of the host. Of the cases used in the present study only 4 wing transplants implanted in the place of, or near to, the host wing have received an appreciable nerve supply.

MORPHOGENESIS

Free appendages.—Table 1 shows that 49 per cent of the free appendages are complete and normal (see Figs. 1, 5, 6, 7). In 12 other cases, only 1 phalanx is missing, so that 49 cases (23 wings and 26 legs) may be considered as typical differentiations. The pattern of

carpals and tarsals is not considered in detail; they are found to be normal in numerous instances. Since 6 of the 9 "crucial" cases belong in this category, it is conclusively shown that the formation of a typical pattern of skeletal elements and their gross morphogenesis are independent of any indirect influence of the nervous system. This statement must be qualified with regard to joint formation (see p. 373).

Hypodactyly is found in 7 cases. In 6 of them it affects the first digits, which are the shortest in normal wings and legs (Fig. 4). In 2 cases the hypodactyly is combined with a slight hypophalangy on the digits present. The fibula, which is rudimentary in normal legs, is absent in 9 cases, in 8 of which the deficiency is combined with hypodactyly. This hypodactyly concerns the digits on the fibular side in some cases and the digits on the tibial side in others. Nine cases show more severe malformations. It is of interest to note that the radius and ulna, or the tibia and fibula, are here most seriously affected; they are abnormally short and atypical in shape. On the other hand, the femur is always nor-

TABLE 1
MORPHOGENESIS OF TRANSPLANTED LIMB SKELETONS

Condition	Wings	Legs	Total	Percentage
Complete and normal	17	20	37	49
Complete, except absence of one phalanx	6	6	12	16
Hypodactyly	4	3	7	9
Absence of radius or fibula, otherwise normal	1	1	2	2
Absence of fibula or ulna, combined with hypodactyly	1	8	9	12
Malformations of higher degree	7	2	9	12
Totals	36	40	76	100

mal, and the humerus is normal in all but 2 cases. These defects may or may not be combined with hypodactyly and hypophalangy. In several cases the long bones are abnormally curved, the metacarpals or the metatarsals, respectively, being affected most frequently. One of the malformed legs resembles the chondrodystrophic condition which is described for the chick in a nonhereditary and a hereditary form by Landauer (1927, 1931). The femur is normal and straight; the tibia is extremely curved and shows hypertrophy of bone on the concave side; the fibula is abnormally strong and of the length of the tibia; both tibia and fibula are fused at their two ends; and one metatarsal bone is curved. A survey of our complete material shows that, although transplantation may interfere with morphogenesis, the resulting malformations are ordinarily not of this type. It seems likely, therefore, that the donor embryo of this particular transplant was incidentally chondrodystrophic.

Duplications are rare. They concern the distal parts only; we find duplications of 1 or 2 phalanges in 3 cases, and of metacarpals and phalanges in 2 cases. One of the latter duplications is in a host wing, whose development apparently has been interfered with by the transplant.

It is obvious that the distal parts are more frequently affected than the proximal parts, and a definite sequence in the deficiencies in distal-proximal direction is observed. Distal

elements, particularly the phalanges, may be abnormal or absent in the presence of normal proximal parts, but absence or abnormality of the latter is generally correlated with defects in the distal parts. No such regularity is found, however, in the pre-postaxial direction. Preaxial elements are affected alone or together with postaxial elements, and vice versa. The radius or tibia may be imperfect or absent in the presence of its normal partner, and vice versa. We conclude that from the stage of transplantation of the bud onward, there are no reciprocal causal relations between preaxial and postaxial elements, nor is there a causal subordination of both to a dominant factor.

The causation of the malformations is obscure. The following factors are excluded as causal agents: stage of the limb buds at transplantation, the site of the transplant (whether in the flank or in the coelom), the axial orientation of the transplant, and the innervation of the transplant. In a few instances concerning purely postaxial deficiencies, the defect may be explained on the assumption that the bud was cut short on its posterior edge. The majority of distal anomalies, however, cannot be explained in a similar way. In a few instances a host limb, developing immediately adjacent to the transplant, interfered with the development of the latter. The malformations must be considered as an inhibition of development resulting from general nonspecific factors which are connected with the operation (such as delayed or insufficient blood supply, etc.).

Pectoral girdles.—Since methylene blue is a cartilage stain, the clavicle is not stained. No data on it are available from our experiments. It is found that in all cases but one the girdle is present. Both scapula and coracoid are present in 32 cases (Figs. 5, 7*b*), the scapula being absent in the remaining 3 cases. In practically every specimen, however, these girdles are reduced greatly, both in absolute size and in proportion to the transplanted limb; in most cases they are "miniature" girdles (cf. Figs. 7*a* and 7*b*). Actual measurements are given below (p. 372).

The two elements of the pectoral girdle are recognizable in every case, although abnormalities in shape are very common and much more frequent than in the free limbs. The scapula is affected in relatively more cases than the coracoid. In most cases, the scapula and coracoid join in the formation of a glenoid cavity, and they meet at the typical angle. In cases in which the transplanted girdle is located near the host sternum, its coracoid articulates with the latter. In several cases in which host and transplant girdles are lying close together, elements of the transplant are fused with those of the host or cartilaginous bridges connect the two. It is found that in several cases one of the two elements is abnormal in shape, while the other is normal. It seems as if each element is self-differentiating with respect to the other, from the time of transplantation on.

Pelvic girdles.—Complete girdles are present in 29 of a total of 40 cases (Figs. 1*b*, 4, 5). At least 2 elements are present in every case. The ischium is absent in only 1 instance, the pubis is absent in 2 cases, and the ilium is absent in 8 cases. The reduction in size and the abnormality in shape of the elements are even more marked than in the pectoral girdle (cf. Fig. 1, *a* and *b*, also Fig. 4). The ilium is most highly affected; in many cases, it is a normally shaped, flat, broad plate, extending anteriorly and posteriorly from the acetabulum, but often its outline is atypical. In some cases it does not extend beyond the acetabulum. The ischium is occasionally a narrow, rodlike structure rather than a broad, flat plate. The pubis is found to be more normal in size and structure than the other 2 elements. All 3 elements join in the formation of the acetabulum and are found in a typical position relative to one another. Fusions with adjacent parts of the host skeleton are very infrequent (in Fig. 6, the ilia of host and transplant are fused). The 3 elements vary

independently of one another with respect to presence or shape. No causal relationship seems to exist in their differentiation; nor do we find a correlation between the degree of differentiation in the free limb and the girdle.

It is difficult to interpret the size reduction and abnormal shape of the girdles,² since very little information is available concerning the localization of the girdle-forming primordia in the stages of operation. At least part of the material for all elements must be located in the transplanted limb primordium, i.e., in the somatopleure lateral to the somites (somites and entoderm are excluded from the transplant). The deficiencies may be explained in one of the following ways: Girdle material may have been destroyed at operation, or part of the girdle-forming area may be located outside of the transplant, and no adequate regulation has taken place. Warren (1934) in experiments of removal of the anterior or posterior half of the wing bud shows that girdle elements are at least partly located in the wing bud proper and that no regulation takes place. His operations, however, were made on limb buds of more advanced stages. We have definite information for one element, the ilium. Its primordium is located partly in the transplant and partly in embryonic structures median to it. In a few cases the donors of leg transplants were raised; they contain fragments of the ilia in their sacral regions; and the corresponding transplants likewise contain deficient ilia. A similar observation was made by Spurling (1923). Measurements support this contention; the ilia are disproportionately small as compared to ischia and elements of the free leg. The same holds for the average size of the transplanted scapulae as compared to the coracoids which are reduced in proportion to the free wings. This indicates that part of the scapula material is located outside of the wing bud proper. Another factor may have an adverse effect on girdle formation: most of the girdles, in contrast to the free limbs, develop inside of structures of the host body and may be exposed to pressure by neighboring tissues.

GROWTH

Altogether, 14 free wing and 9 leg transplants are exactly or nearly exactly of the same size as the corresponding limbs of the host embryo. The great majority of transplants are, however, smaller than the host limbs. In order to obtain precise figures for the growth relations the greatest lengths of the different elements of the girdles and of the free limbs are measured in transplants and in host limbs, and the measurements of the transplants compared with those of their own host, and the ratios then averaged. Altogether, 32 wings and 26 legs are used; the malformed transplants are excluded. The observations include transplants ranging from 8 to 17 days of incubation. No significant differences in relative length are found at different age levels.

Table 2 shows that most elements are reduced 20-30 per cent, whereas the dorsal girdle elements (scapula and ilium) are reduced over 50 per cent. The data indicate that in the wing the metacarpals are more highly affected than other elements, while in the leg the tibia is most highly affected. Our data, however, are not sufficient to establish definitely a differential growth inhibition of these elements.

Ratios within the transplants.—The excessive shortness of scapula and ilium finds its expression in the ratios of scapula:humerus; scapula:coracoid; ilium:femur; and ilium:ischium which are computed for each transplant and its host and then averaged (Table 3). Finally, the proportions of the proximal elements within the free limbs are calculated

² The illustrations of Murray (1926) and of Hunt (1932) show the same deficiency in girdle development in chorio-allantoic grafts.

for transplants and for hosts. The average ratio of humerus:radius is 1.01 for the host wings and 1.16 for the transplant wings, which is not a significant difference. The average ratio of femur:tibia is 0.79 for the host leg and 0.99 for the transplant, which emphasizes again the considerable shortening of the tibia.

The growth reduction of transplants may be due either to an inadequate establishment of vascularization of the transplants or to deficiencies in the innervation. The latter may act in a twofold way, either by controlling the contraction and expansion of blood vessels or by exerting a direct trophic effect on the growing limb bud and on its innervated structures. Two of the wings and one of the legs which had reached the full size of their host limbs are located in a position in which they cannot have received adequate innervation. To judge from these cases, vascularization rather than innervation seems to be the decisive factor in growth control of transplants.

TABLE 2

COMPARISON OF LENGTH RATIOS OF TRANSPLANT AND HOST ELEMENTS .

Trpl. Host	Hum. Hum.	Rad. Rad.	Metacar. Metacar.	Scap. Scap.	Cor. Cor.	Fem. Fem.	Tib. Tib.	Metatar. Metatar.	Il. Il	Isch. Isch.
Averages	0.78	0.77	0.71	0.42	0.71	0.80	0.70	0.80	0.46	0.61

TABLE 3

COMPARISON OF LENGTH RATIOS WITHIN THE HOST AND TRANSPLANT LIMBS

	Scap. Hum.	Scap. Cor.	Hum. Rad.	Il. Fem.	Il. Isch.	Fem. Tib.
Host ratio	1.04	1.54	1.01	1.21	1.94	0.79
Transplant ratio	0.60	1.08	1.16	0.70	1.62	0.99

On the other hand, observations on nerveless frog limbs (Hamburger, 1928) point to growth control by the nervous system. In these experiments the lumbrosacral section of the spinal cord was extirpated unilaterally at early stages prior to nerve outgrowth, whereas the limb bud was not disturbed at all. In these cases the ensuing growth reduction of about 12 per cent must be due to a direct or an indirect effect of the lack of innervation, since the operation does not interfere with the vascularization of the limbs.

HISTOLOGICAL DIFFERENTIATION OF CARTILAGE, AND EARLY OSSIFICATION

Chondrogenesis and early osteogenesis of the transplants are found to be absolutely typical and to proceed synchronously with the host limbs. The arrangement of the cartilage cells in the epiphyses and diaphyses (so-called "functional structures") and the formation of three distinctly different zones (irregular cells with a small amount of matrix in the epiphyses; flattened cells arranged in concentric ellipsoid curves; hypertrophied cells in the diaphysis) are normal in the stages preceding ossification. The formation of the periost, of the first perichondrial cylinder of bone around the cartilaginous shaft, of osseous trabeculae, the erosion of diaphysial cartilage, etc., follow in all details the normal

histogenesis of these elements, as described by Fell (1925) (see Figs. 8-12). Deviations from the normal course are: hypertrophy of bone in the concavities of abnormally curved elements (Fig. 12) and certain abnormalities in those joints which are not separated (see below).

We conclude that histological differentiation of cartilage and the first phases of ossification are independent of nerve supply and of functional activity.

THE FORMATION OF JOINTS

Two different aspects must be considered in a study of joint formation in nerveless limbs: the sculpture of the articulating surfaces of the epiphyses and the histogenesis of the joint-forming area, including its segregation or fusion. Our material is not favorable for a study of the former, since in specimens stained with methylene blue the epiphyses are colored only slightly. A fine, distinct line between two adjacent elements, however, indicates the separation which takes place in most joints on the ninth day of incubation. The heads of femora and of humeri are normal in many cases, but in several others they are abnormal and fused with their girdles (Figs. 1*b*, 4, 7*b*). Condyles and other surface elevations are usually smoother than normal. Flatness of protuberances and shallowness of depressions is a general feature, particularly in the long elements (Figs. 2 and 3). The surface details of phalanges seem to be normal in most cases.

Fusions between adjacent elements are found frequently. In nerveless limbs some joints are usually fused, whereas others are separate; no general rule for their occurrence is discovered. Fusions of all joints are found in only two cases of highly malformed transplants. The joints between long elements seem to be more frequently affected than those between phalanges. Fusions are never found in cases which are known to be innervated.

Adjacent elements usually are not fused along the entire articulating surfaces but at one corner only. This obviously results from the posture in which a nonmotile limb develops. All nerveless and poorly innervated limb transplants, which are paralyzed throughout morphogenesis, develop in a characteristic posture which may be called "static posture." In the case of the wing this corresponds to the posture assumed at rest (Fig. 5). In the leg we find a partial flexure of the metatarsus against the tibia and fibula and a slight flexure of the phalanges (Figs. 4, 8). The "static posture" is apparently the result of differential growth of the skeletal, and possibly also of the muscle, primordia.

In such legs femur and tibia are in close approximation at the innermost point of the knee joint, whereas they are quite separate at the outermost point, near the patella. The inner points are consequently preferential areas of fusion (Fig. 9). Likewise, the condyles will be expected to be better developed at the outer aspect than at the inner angle. This is, indeed, the case. The same holds for the articulation of the humerus with the radius and ulna. Similar differences at different sides of a joint are not found in articulations of metatarsals with phalanges and between phalanges as they are lined up in a slightly bent line.

A few typical cases may illustrate our findings:

Tre 634 (Figs. 1 and 2): A right leg developed inside of the coelom (illustrated in Hamburger, 1939, Fig. 12). It is innervated by two small branches of nerves 16 and 17. The transplant (at operation) was a small bud (stage 4, 64 hours) and was fixed at 18 days of incubation. The femur is fused with the pelvic girdle, but all other joints are separate, and all other articulating surfaces

are approximately normal in their gross structures. The relative smallness of the condyles at the distal end of the tibia and the shallowness of the intercondyloid fossa illustrates well the deficiencies usually found in nonfunctional limbs (Fig. 2).

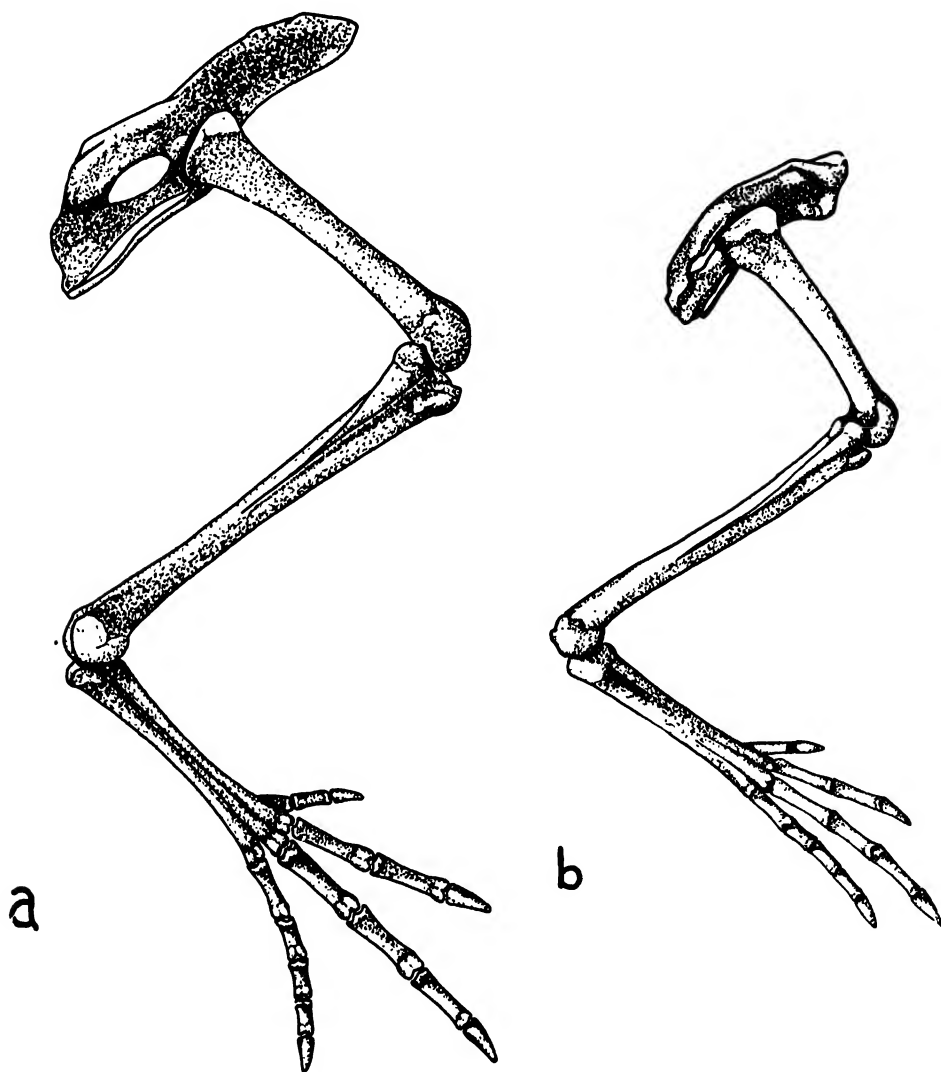


FIG. 1.—(a) *Tre 634*: right leg of host embryo; fixation at 18 days of incubation; dissection; 2.3 \times .
(b) Same: transplant (right leg) developed inside of the coelomic cavity; poorly innervated; dissection; 2.3 \times .

Tre 629 (Fig. 3b): A right wing developed on the umbilical cord and was definitely nerveless (illustrated in Hamburger, 1939, Fig. 10). The transplant (at operation) was a very small bud (stage 3, 68 hours) and was fixed at 19 days of incubation. All skeletal elements are normal.

The head of the humerus is abnormally small, not quite typical in shape, and fused with the rudimentary pectoral girdle. All long elements are likewise fused. No articular condyles are developed at the distal end of the humerus (Fig. 3*b*). The phalanges are present and separate; their articulating surfaces are normal.

Tre 623 (Fig. 4): A left hind limb developed inside of the coelom and was attached to the inside of the body wall by a narrow stalk. It was probably nerveless. The transplant (at operation) was a very small bud (stage 3, 68 hours) and was fixed at 17 days of incubation. The first toe is missing. The girdle and all long elements are fused, and all articulating surfaces are poorly sculptured or absent. All phalanges with the exception of one pair are separate.

Tre 597 (Fig. 5): A right wing developed on the umbilical cord and was definitely nerveless (illustrated in Hamburger, 1938, Fig. 4). The transplant (at operation) was a thickening (stage 1, 52 hours) and was fixed at 9 days of incubation. All elements are normal and only slightly fused or separate.

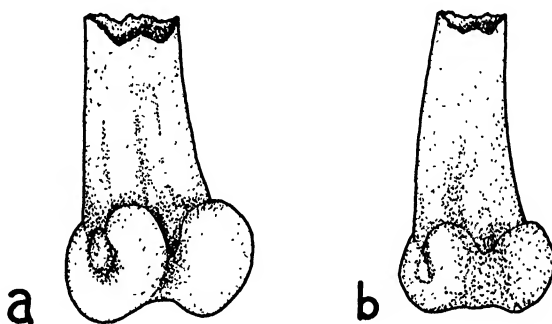


FIG. 2.—Same specimen as Fig. 1. (a) The condyles at the distal end of the tibia of the right host leg; 7 \times . (b) The condyles at the distal end of the tibia of the transplant; 7 \times .

Tre 367 (Fig. 7*b*): A right wing developed on the right flank near the midventral line; it was very poorly or not at all innervated. The transplant (at operation) was a small bud (stage 4) and was fixed at 14 days of incubation. A fusion is found between humerus and radius and ulna; all other joints are separate, and their articulating surfaces are normal. In particular, the head of the humerus which is much too large for its glenoid cavity shows a sculpture typical for its stage of development.

Tre 371 (Fig. 6): A right hind limb developed in the flank in front of the right host leg and was probably functionally innervated. The transplant (at operation) was a small bud (stage 4) and was fixed at 13 days of incubation. The pelvic girdles of host and transplant are fused. The transplant is perfectly normal in every respect.

A study of sections and of specimens stained with methylene blue shows that two different types of fusion must be distinguished, one in which the epiphyses are histologically normal and clearly demarcated but united by a small area of fibrous connective tissue and another in which adjacent elements are connected by cartilaginous tissue which has obscured the border line between the two elements. This latter mode of fusion is rare, whereas the former type is frequent. In specimens stained in methylene blue fusion by cartilage is indicated by continuity of the deep blue stain (e.g., Fig. 4).

The two types may be readily explained on the basis of our knowledge of normal joint formation. The histogenesis of the avian knee joint is described by Fell and Canti (1934). The knee-joint region consists of a dense mass of undifferentiated "mesoderm" at a stage

when femur, tibia, and fibula begin to chondrify at their diaphyses. The direct continuity between the three elements persists for some time, and in a 5-day embryo "in thionin-stained preparations it is interesting to note that cartilage matrix of a very young and

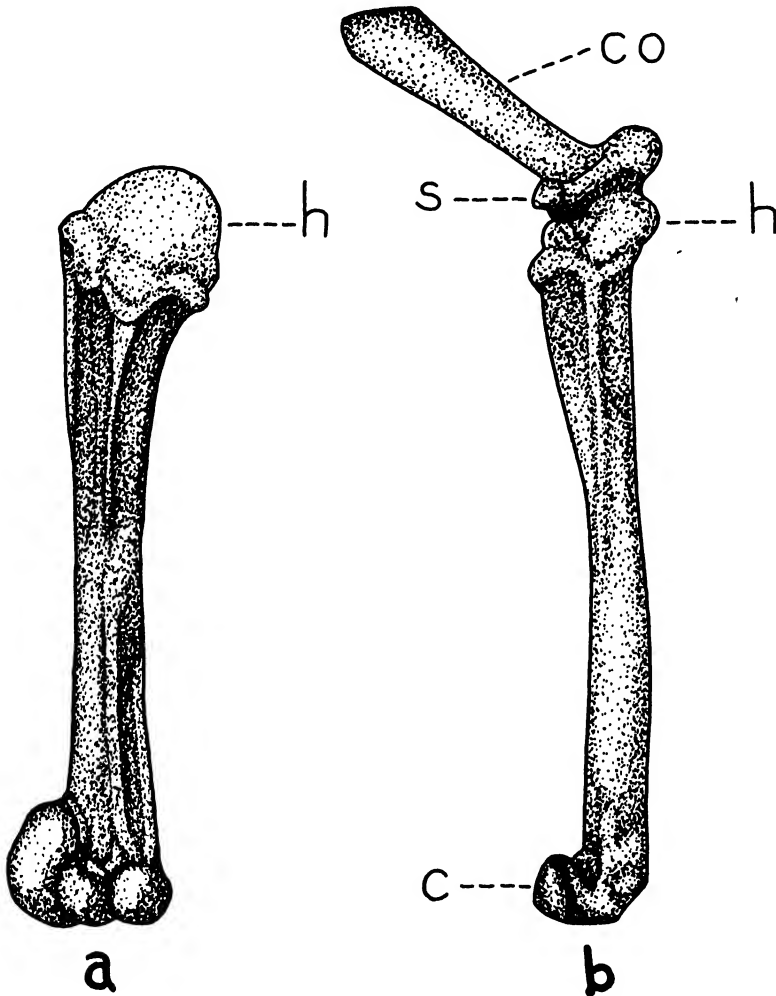


FIG. 3.—(a) *Tre 629*: right humerus of host; fixation at 19 days of incubation; dissection; 10X. (b) Same: humerus and rudimentary pectoral girdle of transplant (right wing) developed on the umbilical cord; nerveless; dissection; 10X; the humerus was fused with the radius and ulna and was severed at the level of the joint at *c*; *co*=coracoid; *h*=head of humerus; *s*=scapula.

immature type extends right across the line of the joint . . . " (Fell and Canti, 1934, p. 320). Cartilage matrix is still visible in the joint area of the 6-day embryo, but about the eighth day it is replaced gradually by, or transformed into, loose fibrous tissue in

which a cleft appears. The loose tissue disappears eventually. According to our observations, joint formation is essentially similar in the other joints.

The first, more frequent, type of fusion (by connective tissue) is explained readily by assuming that joint formation proceeds normally but stops short of the final step—the segregation of adjoining elements. This type we may consider as a “primary fusion,” or “nonfission” (Warren, 1934). In the second type of fusion (by cartilage) we assume either that the normally transitory phase of cartilaginous differentiation in the joint persists instead of undergoing regressive changes or that fibrous joint tissue is formed first but replaced later by cartilage in a second atypical phase of chondrification. This latter interpretation which must be designated as a “secondary fusion” by cartilage is probably correct. Fell and Canti (1934) found in tissue cultures of knee-joint regions exactly this sequence of events—the formation of a marked articular line of dense connective tissue by the fourth day of cultivation, i.e., at a total age of 8 days.

After 6 days cultivation *in vitro*, further joint differentiation ceases and actual fusion of the bone rudiments takes place. This is shown in the living culture by the fact that the dark line across the knee joint becomes less and less distinct. . . . Sections of an explant cultivated for 17 days show direct continuity between the three cartilages at their points of contact [*op. cit.*, p. 324].

This the authors call “secondary fusion.” The fact that in our material primary fusions are frequent and secondary fusions rare supports further this interpretation. Occasionally the same joint shows both types of fusion. One knee joint (Fig. 8) is found to be separate at the outer aspect, i.e., in the region of the patella. Primary fusion of femur and tibia by fibrous tissue is found in the middle region (Fig. 9) and secondary fusion by cartilage in the inner angle (Fig. 10).

Such observations suggest that growth pressure or other mechanical conditions at the point where opposed elements meet each other are responsible for secondary cartilage formation. Glücksmann (1939) demonstrated the transformation of fibrous tissue into cartilage under similar conditions of slight pressure in tissue-culture experiments. No sectioned cases older than 12 days are available. It will be of interest to find out if a secondary cartilage fusion is more frequent in later stages than in younger, i.e., whether the process of secondary fusion is progressive during development.

DISCUSSION

The present material demonstrates to what a remarkable degree of perfection the differentiation of the limb skeleton of the chick may proceed in the absence of innervation and of function. The method of intra-embryonic transplantation provides for optimal conditions and, therefore, reveals the inherent potencies of the transplants to their fullest extent. In previous isolation experiments of limb primordia of about the same stages (48–72 hours of incubation) in which the method of chorio-allantoic grafting was used (Murray and Huxley, 1925; Murray, 1926, 1928; Hunt, 1932) abnormal skeletons were obtained, in which many elements were distorted or missing and many were fused. It is now obvious that these deficiencies, with the exception of the fusions, are due not to a lack of self-differentiating potencies of the transplants but to spatial restrictions and other unfavorable developmental conditions on the chorio-allantoic membrane. The failure of 2-day primordia to differentiate into any structures resembling a normal skeleton

(Murray, 1928) shows merely that these young stages are more susceptible to the unfavorable conditions of the membrane than are older stages.

Functional activity and joint formation.—Even the best-developed cases of our nerveless or poorly innervated transplants show more or less severe defects in joint formation. Primary and secondary fusions (see p. 374) are common, particularly in the joints of girdles and between long bones. Other joints, particularly those between phalanges, may be completely separate. The fact that by no means all joints of a nerveless limb are necessarily fused indicates that the embryonic movements are at best a subsidiary factor in bringing about separation. At present we have no explanation for the observation that some joints are fused while others are not. Moreover, it was pointed out heretofore (p. 377) that movements can be instrumental only in bringing about the final step in joint formation—the formation of a cleft; whereas all preceding steps (differentiation of the articular cartilages, of fibrous articular tissue between the articular cartilages, etc.) proceed normally, not only in our transplants but even *in vitro* (Fell and Canti, 1934). In addition to fusions, deficiencies in the sculptural details of articulating surfaces are common; condyles are smoother, and grooves are shallower than normal.

These observations are in full agreement with earlier experiments on nerveless limbs in anurans and in the chick. Of the crucial experiments we mention those of Braus (1909, 1910), who transplanted fore-limb buds of *Bombinator* heterotopically and found that the girdles were abnormally small and the limbs immobilized. The joint separation was complete, but the head of the humerus, which did not fit into the glenoid cavity, showed deficiencies in its surface structure. In our own experiments on nerveless limbs of frogs (Hamburger, 1928) it was observed that a separation of joints was usual, but fusions occurred occasionally, and minor details of the surface sculpture were deficient. For the chick we mention the extensive chorio-allantoic grafting experiments of Murray (1926, 1928), whose best-developed cases proceed far toward normality. Finally, the tissue-culture experiments of Fell and Canti (1934) showed that the presumptive knee-joint area of a leg bud of a 4-day embryo when isolated proceeded to almost complete joint formation but stopped short of the final step, the separation of femur and tibia. Murray and Huxley (1925) and Murray and Selby (1930) carried the analysis a step further by showing that an articulating surface will self-differentiate nearly to perfection even in the absence of the contiguous element. Isolated primordia of humeri and femora were reared on the chorio-allantoic membrane. All typical parts (head, condyles, etc.) were formed, but the same imperfections of surface details were observed as in our experiments. Fusions of contiguous elements were also observed by Warren (1934) in experiments, in which halves of wing buds were allowed to differentiate *in situ*, and by Schmalhausen (1925) and by Brunst (1927, 1932) in regenerating limbs of salamanders which were paralyzed by nerve resection.

The role which innervation plays in joint formation is a minor one; yet it is desirable to analyze the mechanism by which it enters into this complex process. It may be operative in one of the following ways. (1) Lack of innervation results in a complete paralysis of the limb throughout development. The occasional nonfusion of adjacent elements may be ascribed to this immobilization. Slow gliding movements are possibly a subsidiary factor in bringing about the separation which is thus doubly assured. (2) The deficiencies in sculptural details we are inclined to explain in a different way. The absence of innervation results in an atrophy and degeneration of the skeletal musculature, which is much more radical in the chick than in the frog (Hamburger, 1928). It is likely that the tendi-

nous attachments of such muscles are structurally deficient, and it is certain that no pull is ever exerted on these points of attachment. It is conceivable that the formation of protuberances, i.e., local growth of cartilage or local bone deposition, requires normal and functionally active attachments of tendons and ligaments, i.e., the exertion of pull and stress. The importance of such factors in the mechanism of chondrogenesis is clearly brought out by Glücksmann (1939). (3) The possibility that these abnormalities may be caused by disturbances in the metabolism of the skeletogenous tissue should not be lost sight of. The nervous system may exert an indirect trophic control on skeletogenesis. It is our experience as well as that of other investigators that skeletons which are otherwise highly abnormal show an increased tendency toward fusions. Fell and Canti (1934) and Fell and Landauer (1935) observed that fusions of tibia and fibula were frequent *in vitro* and more marked if a special growth-restricting medium were used. Schmalhausen (1925) found fusions of cartilages in the limbs of salamanders which had been starved or exposed to high temperatures during morphogenesis. The experimental evidence available does not permit us to decide between these three alternatives. We wish to point out, however, that from the point of view of developmental mechanics "functional activity" covers a complex of heterogeneous factors in skeletogenesis.

Whereas the primary development of the limb skeleton is thus shown to be self-differentiation to a high degree, extrinsic factors become of increasing importance in later phases of bone development. It is well known that the details of articulating surfaces, and, in particular, the "trajectorial" arrangement of the trabecles—are formed under the influence of external mechanical conditions to which they are exposed (tensions, stresses, etc.) and that structural transformations are brought about easily as a response to changed mechanical factors, e.g., in fractured bones. These findings underscore Harrison's warning (1933) against the indiscriminate acceptance of the concept that differentiation proceeds universally from an undetermined state to one of rigid mosaic development.

SUMMARY

Nerveless and poorly innervated wings and legs of chick embryos are obtained by transplantation of early primordia to a ventral position, into the coelomic cavity, or to the umbilical cord. Investigations of the skeleton of the transplants, 8–18 days of age, in whole-mount preparations and in sections gives the following results:

1. Morphogenesis in the absence of innervation is normal in a high percentage of cases. Hypodactyly and hypophalangy are the most common abnormalities in transplants.

2. Chondrification and the first phases of ossification proceed normally in the absence of innervation.

3. Joint formation in nerveless limbs may be almost perfect and complete. Smoothness of articulating surfaces and fusions of joints, however, are found in the majority of cases. Two types of fusion, primary and secondary, are distinguished, and their origin is discussed.

4. Skeletal growth of transplants is reduced approximately 20 per cent. A possible trophic effect of the nervous system is discussed.

5. The girdles are more poorly developed than are the free limbs. Particularly the dorsal girdle elements, the scapula, and the ilium, are disproportionately small. This result makes it likely that the primordia of scapula and ilium are located in part outside of the transplanted primordium.

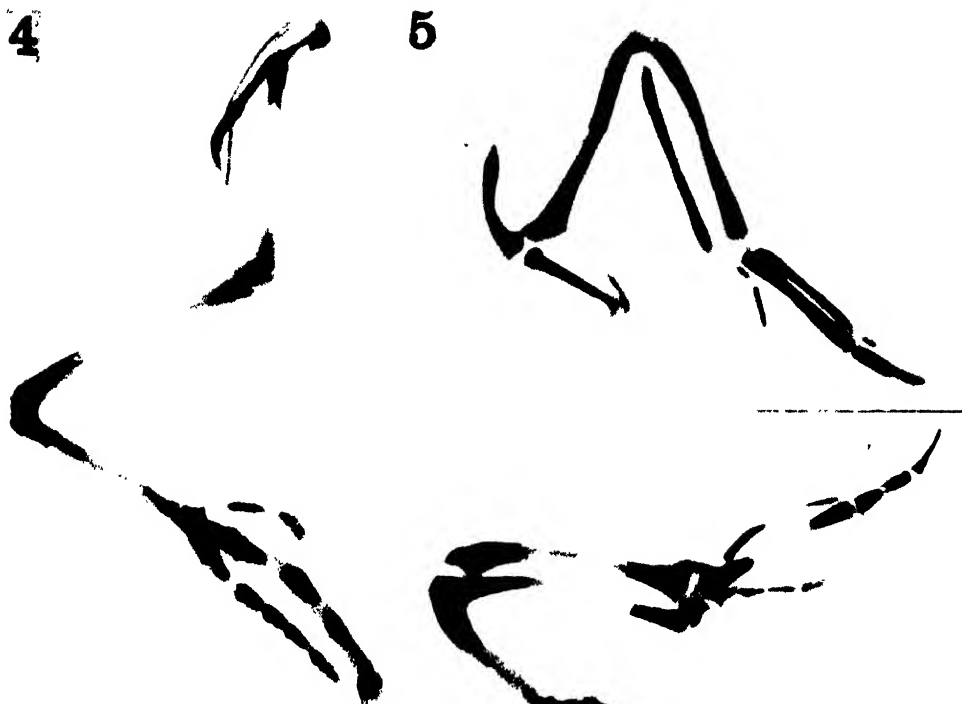
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PLATE I

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7a



PLATE I

FIG. 4.—*Tre 623*: Transplant (left leg) developed inside of coelomic cavity, nerveless; fixation at 17 days of incubation; methylene blue; $3\times$.

FIG. 5.—*Tre 597*: Transplant (right wing) developed on the umbilical cord; nerveless; fixation at 9 days of incubation; methylene blue; $7.5\times$.

FIG. 6.—*Tre 371*: Transplant (right leg) developed on right flank; probably innervated; ventral view; fixation at 13 days of incubation; methylene blue; $2.5\times$; *p*=pubis of transplant; *t*=transplant.

FIG. 7*a*.—*Tre 367*: Right host wing and pectoral girdle, disarticulated; fixation at 14 days of incubation; methylene blue; $4.3\times$.

FIG. 7*b*.—Same: Transplant (right wing) developed on right flank near ventral midline; probably poorly or not innervated; methylene blue; $4.3\times$; *co*=coracoid; *s*=scapula.

PLATE II

FIG. 8.—*Tre 615*: Transplant (right leg) developed on the umbilical cord; nerveless; fixation at 12 days of incubation; Heidenhain's hematoxylin; 7 \times ; *k*=knee joint.

FIG. 9.—Same: Knee joint; primary fusion (see text); 27 \times .

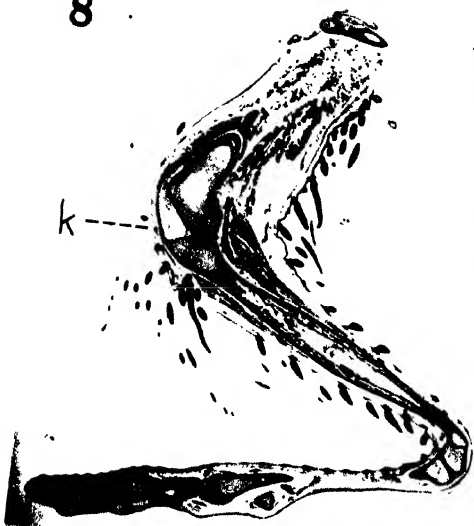
FIG. 10.—Same: Knee joint; secondary (cartilage) fusion; section median to Fig. 9; 27 \times .

FIG. 11.—Knee joint of normal 9-day embryo; Heidenhain's hematoxylin; 27 \times .

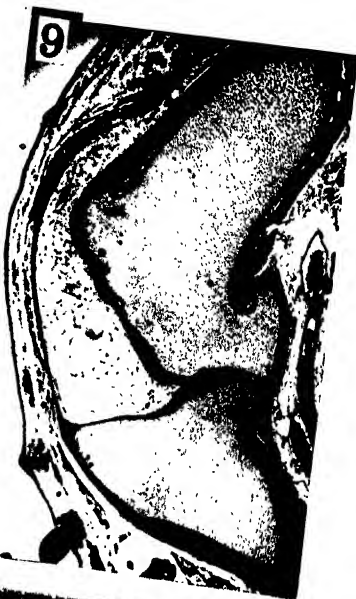
FIG. 12.—*Tre 572*: Transplant (right leg) developed in coelomic cavity; nerveless; longitudinal section through femur and knee joint; fixation at 10 days of incubation; Heidenhain's hematoxylin; 16 \times .

PLATE II

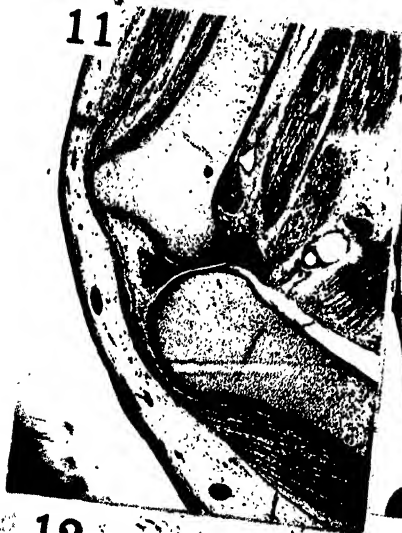
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THE STIMULUS FOR MATURATION AND FOR OVULATION OF THE FROG'S EGG

(One plate)

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IT IS well known that many Amphibia will ovulate in response to injections of anterior lobe pituitary gland from the same species. Such ovulation is normal, and during release and passage of the egg through the oviduct maturation changes occur leading to formation of the first polar body and the second maturation spindle, after which fertilization is possible. In *Rana* ovulation can be produced by pituitary from most Anura and sometimes by large amounts of *Triturus* pituitary, mammalian pituitary extracts, and human pregnancy urine (see review by Creaser and Gorbman, 1939). *Xenopus laevis* responds consistently to large doses of mammalian pituitary and human pregnancy urine, also to progesterone and several other steroids (Bellerby, 1933; Zwarenstein, 1937; Shapiro, 1936). The injected pituitary appears to be able to act directly upon the ovary, for ovulation can be induced after removal of the host pituitary (although under such circumstances there may be quantitative alterations in the reaction) (Novelli, 1932; Canturivili, 1936; Wunder, 1938b; Rugh, 1939) or of many other organs, including the thyroid and adrenals (Novelli), and also after destruction of the central nervous system by "pithing." Heilbrunn, Daugherty, and Wilbur (1939) found that excised frog ovaries will ovulate in suspensions of frog pituitary but not in plain Ringer's or in muscle-tissue suspensions (one experiment). Also Zwarenstein (1937) obtained ovulation in excised ovaries of *Xenopus* with progesterone suspensions.

Thus there is no evidence to suggest the involvement of any organ other than the pituitary and ovary in ovulation of the frog's egg, but some suggestion that a specific pituitary stimulus is not required. Evidence regarding maturation of the egg is less clear. Heilbrunn, Daugherty, and Wilbur report maturation of eggs shed by excised ovaries, but their data concern nuclear breakdown, which is not a very demanding criterion. Likewise, their photographs are not convincing. Zwarenstein found that eggs released by progesterone from normal animals were fertilizable, but no data regarding eggs shed by hypophysectomized animals or excised ovaries are given.

PROBLEM

Since the excised ovary affords a better opportunity than does the intact animal for controlled experimentation, with certainty as to the direct application of stimuli to the ovary itself, this method would appear to be highly favorable for investigation of the mechanisms involved in maturation and ovulation of the egg. However, it is essential to be sure that the changes induced *in vitro* are identical with those occurring *in vivo* under pituitary stimulation and in spontaneous ovulation. The experiments here reported were performed to determine whether this is so and also to determine whether both ovulation and maturation are initiated by a specific substance confined, in *Rana*, to the pituitary gland.

PROCEDURE

The experimental procedure used was essentially that of Heilbrunn, Daugherty, and Wilbur. All the animals were *Rana pipiens brachycephala* Cope (Stejneger and Barbour, 1939) from upper Vermont and the St. Lawrence Valley. Most of the experiments were on animals taken from hibernation in April, but a second series of experiments was carried out in November. Fragments of ovary, each containing about 100 eggs, were first rinsed in Ringer's and then immersed in a suspension made by mashing either one anterior lobe pituitary or about an equal volume of another tissue in 30 cc. of Ringer's. Other control fragments were kept in plain Ringer's. In each experiment parts of a single ovary were used in parallel pituitary and control preparations. In some cases whole ovaries were used with 2 pituitaries in 30 cc. of Ringer's, the other ovary being treated with an equal or larger amount of another tissue. All experiments were conducted at about 22° C. At intervals of about 6 hours ovulated eggs were counted and fixed in Smith's fluid. The fragment or ovary remaining after ovulation had stopped was preserved in the same way.

OVULATION

Table 1 summarizes the results of the spring experiments. A significant fact emerging is that a number of tissues other than pituitary caused some ovulation. Clearly, pituitary is the most potent and the only one which gave consistent results, but ovulation

TABLE 1

OVULATION *in vitro*: SPRING ANIMALS

Tissue	Number of Experiments	Number of Positive Results	Mean Percentage of Eggs Shed in Positive Cases (Approx.)	Tissue	Number of Experiments	Number of Positive Results	Mean Percentage of Eggs Shed in Positive Cases (Approx.)
Pituitary	10	10	12	Stomach	4	0	0
Pituitary* . . .	8	8	8	Blood	3	0	0
Muscle	7	1	15	Liver	2	0	0
Medulla*	5	2	2	Heart	2	0	0
Cerebrum	2	0	0	Pancreas	2	0	0
Cerebrum*	2	1	2	Intestine	2	0	0
Adrenal	4	2	2	Ovary	1	0	0
Kidney	2	1	3	Ringer's	5	0	0
Testis	2	1	2				

* Whole ovaries; more control tissue than pituitary.

was obtained with 6 out of 13 other tissues tried. The number of experiments was too small to exclude the possibility that other tissues might sometimes be effective. That ovulation was due to the tissue present and not to spontaneous ovulation of the excised ovaries is shown by the following facts: (a) about 100 females from the same batch of animals kept in the laboratory failed to ovulate spontaneously; (b) control fragments of the same ovaries kept in plain Ringer's did not ovulate; (c) unattached fragments of ovary, when left in the body cavity, do not shed their eggs. Three experiments performed with pituitary on ovary fragments in early October were negative, but at this season even

intact animals often fail to respond to pituitary injections. Six pituitary experiments performed during November were all positive. Since the ovary is less sensitive to the ovulation-inducing substance of the pituitary in November than in April (Wunder, 1938a) it is not surprising that muscle and medulla failed to induce ovulation in the autumn.

In 4 November experiments the concentration of pituitary was varied. For each experiment a suspension of female pituitaries was prepared and repeatedly diluted to give preparations containing from 4 pituitaries to $1/128$ of a pituitary in 10 cc. of Ringer's. A single ovary was divided into pieces containing 5-10 eggs; and about 125 eggs, taken at random, were put into each preparation. Possible regional variations in the sensitivity of the ovary were thus eliminated so far as possible. Dilutions containing $1/4$ or less of a pituitary in 10 cc. failed to cause ovulation; the results with greater concentrations

TABLE 2
CONCENTRATION AND OVULATION

EXPERIMENT		AMOUNT OF FEMALE PITUITARY IN 10 CC.							
		4	2	1	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$
I	{ Eggs used	226	204	197	179	192	172
	{ Percentage shed	22.6	10.8	15.2	11.7	1.6	3.5
II	{ Eggs used	122	103	113	85	104	110
	{ Percentage shed	13.1	23.3	32.7	18.8	18.3	32.7
III	{ Eggs used	94	87	98	74	99	86	88
	{ Percentage shed	22.4	26.4	55.2	68.9	43.5	59.3	5.8
IV	{ Eggs used	150	154	135	131	148	141	124
	{ Percentage shed	19.6	55.8	34.8	16.8	30.4	29.8	3.2
Total eggs used		122	573	558	515	488	549	399	212
Mean percentage shed		13.1	21.8	30.1	28.5	23.1	23.1	24.8	4.3

are summarized in Table 2. It will be seen that there is a decrease in response at both high and low concentrations. The mean optimum concentration was about one pituitary in 10 cc., but the optimum was perhaps different in each experiment. When a statistical test for effectiveness of all the active concentrations was made, a significant variation arising from the concentration differences was found ($\chi^2 = 56.2$; $n = 6$; $P < 0.01$). When classes 2 and $\frac{1}{4}$ were contrasted with the optimum classes 1 and $\frac{1}{2}$ by means of a fourfold table, ovulation was found to be significantly less in the former ($\chi^2 = 7.84$; $n = 1$; $P < 0.01$). Thus, for November frogs under these experimental conditions, there is an optimum concentration, represented by $\frac{1}{2}$ -1 pituitary in 10 cc. of Ringer's, above and below which the ovulation response *in vitro* is reduced. *In vivo* experiments have not shown that ovulation decreases when an excess of pituitary is injected. However, it must be noted that this effect is not seen *in vitro* until sixty-four times the minimum effective amount is used, i.e., an amount corresponding to about 128 pituitaries for an intact animal.

The amount of ovulation obtained was always considerably less than that ordinarily

attained *in vivo* (the maximum response was 68.9 per cent,¹ and the mean for November and spring experiments was 23.5 per cent and 12 per cent, respectively). This was apparently due to disintegrative changes in the ovarian fragments. The first eggs to be released usually fell from the follicles about 10 minutes after their initial rupture. But extrusion became progressively slower, and when eggs had ceased to fall from the ovaries there were always to be found follicles which had ruptured normally but had failed to extrude their eggs. Ovaries stimulated *in vivo* and excised during ovulation similarly fail to shed all their eggs.

NORMALITY

That the process of ovulation observed in excised ovaries is (until impeded by cytolysis of the ovary tissue) essentially identical with that occurring *in vivo* is indicated by the following similarities between them: (1) The duration of the latent period between initial stimulation and initial follicular rupture is similar since ovulation began after 14-19 hours' immersion. (2) Increasing the amount of pituitary used does not noticeably affect the length of this latent period. The number of eggs shed is roughly proportional to the amount of pituitary used in both cases (at least when near the minimum threshold concentration of pituitary). (3) Comparison of ovulation in excised ovaries with that in ovaries stimulated *in vivo* (described by Rugh, 1935) failed to show any difference in the visible characteristics of the process in the two cases. (4) Shedding of eggs invariably took place through the normal point of dehiscence, i.e., to the exterior through the area of attachment of the follicle to the ovary wall. In two experiments a single lobe of an ovary was tied off and pituitary suspension was injected into the cavity of the isolated lobe. The whole ovary was then immersed in 30 cc. of Ringer's. Most of the eggs in the injected lobes were shed and, without exception, to the exterior. No eggs were released from other parts of the ovaries. (5) Maturation of the egg occurs during muscle- and pituitary-induced shedding of eggs from excised ovaries as in normal ovulation (see below).

MATURATION

That maturation changes, essentially identical with those occurring in the intact animal, take place in eggs released from excised ovaries by pituitary has been established by four methods: (1) Three batches of pituitary-released eggs were examined for polar bodies, which were found in all 180 eggs. (2) Fifty eggs released from an isolated lobe of an ovary injected with pituitary were dissected under a binocular microscope, but in no case was an intact germinal vesicle found. Fifty eggs remaining in the uninjected parts of the same ovary all had intact germinal vesicles. (3) Eggs taken from the coelom of an ovulating female, and consequently without oviducal jelly coats, cannot be fertilized. In order to test the fertilizability of eggs from excised ovaries it was thus necessary to provide them with normal jelly coats. About 150 eggs released from an excised, pituitary-treated ovary were placed, 48 hours after the initial stimulation, in the body cavity of a female ovariectomized 48 hours previously (the same animal from which the ovary used had been taken). Twenty-four hours later the eggs were taken from the "uteri" and artificially inseminated. About one-third of the eggs were already cytolysing. The remainder fertilized and began cleavage. Most of them developed through gastrulation, and 39 hatched as swimming larvae. Since a number of the eggs

¹ Complete ovulation has since been obtained in many cases.

had already begun to cytolize at the time of fertilization, it is not surprising that many of the remainder developed abnormally. Cytological study showed that many eggs were abnormal after 2 days in the suspensions (Fig. 5). (4) The cytological methods used proved highly favorable for the study of maturation processes. Smith's fluid fixed spindles in a light-refractive state, making them very obvious in the absence of any counterstain. The Feulgen technique stained chromosomes an intense red, while yolk granules were colored only faintly. In eggs released by pituitary and by muscle suspensions, which were fixed shortly after shedding, cytological figures were obtained showing 13 tetrads dispersed about a spindle which was oriented radially in the egg and at metaphase of the first maturation division (Fig. 1). Anaphase showed 13 dyads in the egg and 13 in the polar-body projection (Fig. 2). In 4 cases the second maturation spindle, with its associated chromosomes, was found beneath the completed first polar body (Figs. 3 and 4). This figure is identical with that found in spontaneously ovulated eggs taken from the "uterus." Eggs normally remain in this state until fertilized (Parmenter, 1933; Porter, 1939). The stages seen are the same as those observed by E. J. Ryan (unpublished, 1940) for the normal maturation of the eggs of *R. pipiens* and also resemble those described by King (1901) for the toad. Thus, it seems probable that entirely normal maturation took place in at least some of the eggs released *in vitro* by pituitary and by muscle-tissue suspensions.

In 13 out of 138 eggs examined cytologically the germinal vesicle was found to be intact even 40 hours after ovulation (Fig. 6). These eggs included all that had been released by kidney (3), testis (2), and adrenal (4), 1 out of 15 released by muscle, and 3 out of 114 released by pituitary. Eggs released by brain tissues were not examined cytologically. Seemingly true ovulation had occurred in these cases, since all eggs were free from investing follicle membranes. Thus, ovulation can apparently take place independent of maturation.

DISCUSSION

There is thus much evidence that ovulation and maturation induced *in vitro* by means of pituitary suspensions differ in no important respect from the corresponding processes occurring *in vivo*. We are therefore of the opinion that the method described here is suitable for further study of the ovulation and maturation processes in the frog.

Muscle suspensions also can stimulate both processes, at least in highly sensitive spring ovaries. Other tissues can cause ovulation but apparently not maturation. There is some suggestion that these differences may be only quantitative, since maturation did not take place in a few eggs released by pituitary and muscle. Since in *Xenopus* a variety of steroids as well as several gonadotropic preparations can induce ovulation (Shapiro, Zwarenstein), there is no reason to suppose that the ovulation in *Rana*, induced by brain, muscle, adrenal, testis, and kidney suspensions, is due to the same substance that is present in the pituitary extracts. It is evident, however, that, whatever the nature of the ovulation-inducing stimulus, it is not entirely confined in *Rana* to the pituitary gland.

The results of these experiments are consistent with the hypothesis (Rugh, 1935) that the release of the egg is due to action upon the follicular membranes of a digestive enzyme produced by the egg under pituitary stimulation, although the activator need not be an enzyme. In the ligature experiments eggs were shed from the isolated lobes, but not from the rest of the ovary. And the eggs were shed to the exterior, although

the stimulating substance was inclosed in the ovarian cavity. If rupture were due to a diffusible substance which had a differential action on the point of attachment of the follicle to the ovary, it would be difficult to explain the absence of rupture in eggs not isolated but adjacent to the ligature. It might, then, be tentatively suggested that the stimulating substance acts indirectly by means of a mechanism in the egg or follicle. In any event, ovulation could hardly be due to a general histolytic action of the suspension upon follicular tissue, especially since release of eggs ceases when histolysis becomes visible. That ovulation is not due to release of any substance from the germinal vesicle as a result of maturation is indicated by the fact that eggs may sometimes be released without breakdown of the germinal vesicle.

The quantitative methods described provide the basis for a more satisfactory method of comparatively assaying frog pituitaries for ovulation-producing potency than any hitherto available. While there are quantitative differences discernible in the reaction of intact animals to graded doses of pituitary (Rugh, 1937; Wunder, 1938a), there is a great deal of individual variation in the sensitivity of different animals to the same dose of pituitary. Accurate assay by methods based on the intact animal, therefore, requires use of many animals and is possible only when large amounts of pituitary are available for test. Both of these difficulties can be avoided by use of excised ovaries. A single ovary provides ample material for many tests, and regional variation in the sensitivity of the follicles can be eliminated by random selection of eggs used in parallel test and control preparations. The amount of pituitary needed for a test is very small. If the threshold concentration for response were used as a basis for assay, a satisfactory comparison of potency could probably be made between 2 single pituitaries, since 1 pituitary is (in November) enough for about 30 threshold experiments.

SUMMARY

1. Ovulation and maturation of the frog's egg can be induced in excised ovaries by means of anterior lobe pituitary suspensions and sometimes by muscle suspensions. Ovulation can sometimes be induced by suspensions of several other tissues.

2. Evidence is adduced for the contention that ovulation and maturation produced in this way are essentially identical with the same processes induced *in vivo*. The *in vitro* method is therefore considered suitable for further study of ovulation and maturation processes in the frog.

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PLATE I

FIG. 1.—Section of an egg released *in vitro* by muscle suspension. Some of the tetrads on the first maturation spindle are very evident. $\times 750$.

FIG. 2.—Section of an egg released *in vitro* by pituitary suspension. Several dyads are visible in the first polar-lobe projection; the spindle was cut diagonally, and the dyads remaining in the egg are in adjacent sections. $\times 750$.

FIG. 3.—Section of an egg released *in vitro* by pituitary suspension. Chromosomes are seen in the indistinct first polar body. The second maturation spindle is in metaphase, and the chromosomes seem small because they are viewed on end. $\times 750$.

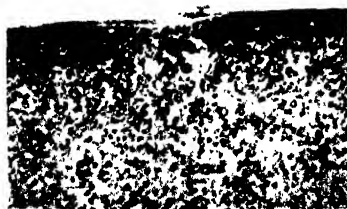
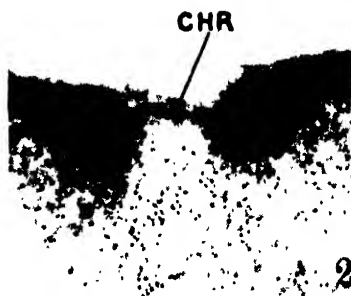
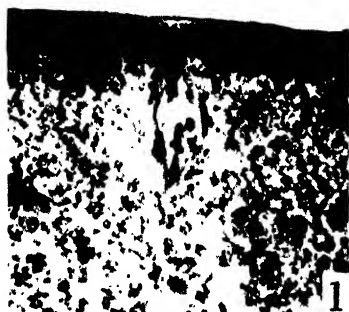
FIG. 4.—Section of an egg released *in vitro* by pituitary suspension. The first polar body is distinct under the egg membrane, and the second maturation spindle is in an adjacent section. $\times 750$.

FIG. 5.—Section of an egg released *in vitro* by pituitary suspension and fixed 48 hours later. The chromatin is clumped at the periphery, and a spindle is not visible. $\times 750$.

FIG. 6.—Section of an egg released *in vitro* by pituitary suspension. The germinal vesicle is still intact; note the absence of follicle cells. $\times 60$.

These eggs were fixed in Smith's fluid and stained by the Feulgen method. Although the chromosomes and pigment granules appear black in the photographs, it must be remembered that in the sections the chromosomes were bright red.

PLATE I



PHYSIOLOGICAL CHARACTERISTICS OF THE DIAPAUSE GRASSHOPPER EGG. I. THE STABILITY OF THE DIAPAUSE CONDITION¹

(Three figures)

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THE mechanism of diapause in the Orthoptera is poorly understood. Bodine (1932) has postulated the existence of a diapause factor x , responsible for the appearance of the resting condition in the egg of the grasshopper, *Melanoplus differentialis*. This developmental and physiological block may be prevented or broken by prolonged exposure to low temperature (Bodine, 1925 and later; Slifer, 1932; Burdick, 1937). Its variation in potency within the eggs of a given pod is shown by the wide range of hatching time for individuals when kept at a constant high temperature (Slifer, 1931; Burdick, 1937).

Detection of changes in the egg of the grasshopper during the diapause period has received little attention. Slifer (1931) states that mitotic division stops rather abruptly within a week after the onset of diapause and the embryonic cells remain in the resting condition until postdiapause development starts. Bodine (1929) showed that the weight of the egg increased only slightly, if at all, from the start of diapause to the one-hundredth day. The mean level of respiration of eggs from the thirtieth to the one hundred and seventy-fifth day remains low (Boell, 1935; Robbie, Boell, and Bodine, 1938). It is the aim of the present investigation to study certain conditions which exist in the egg during the diapause period, in the hope that a better understanding of the regulatory mechanism may result.

The morphological development which follows the breaking of diapause is easily observed. By treating eggs of different ages with agents that tend to break diapause, it is possible to determine the relative potency of the block by noting the number of individuals which are induced to resume development. Experiments of this type are reported in this publication; studies on changes in density, weight, and other characters during diapause will be reported in a further paper.

MATERIAL AND METHODS

Eggs of the grasshopper, *M. differentialis*, were used in all the experiments. Within 24 hours of laying, the pods were collected and placed at 25° C. Particular care was taken to insure satisfactory conditions of aeration and moisture during storage. The entire pods were kept in covered dishes on a firm layer of damp, but not wet, sand, and at intervals of a few days the covers were removed and the pods agitated.

By observing the position of the embryo's pigmented eyes within the egg, it is possible to detect postdiapause development without in any way disturbing the protective mem-

¹ Aided by a grant from the Rockefeller Foundation for work in cellular biology.

branes. To do this more readily, light from a microscope lamp was reflected from a mirror below the stage of a dissecting microscope in such a way that the rays passed obliquely through the eggs (Fig. 1). When observed in this fashion, the pigmented eyes are in conspicuous contrast to the lighter, remaining portions of the embryo and yolk. When the light comes from above, the eggs appear opaque, and it is difficult to see the eyes.

A Petri dish with three paraffin chambers and a narrow connecting channel the width of the low-power dissecting microscope field (Fig. 1) is a help in sorting eggs. When in use, the dish is filled with water, and the eggs are placed in one chamber. As each one is pushed through the field of view in the channel, it is examined and directed to either of the other sections—the satisfactory individuals to one, the discards to another.

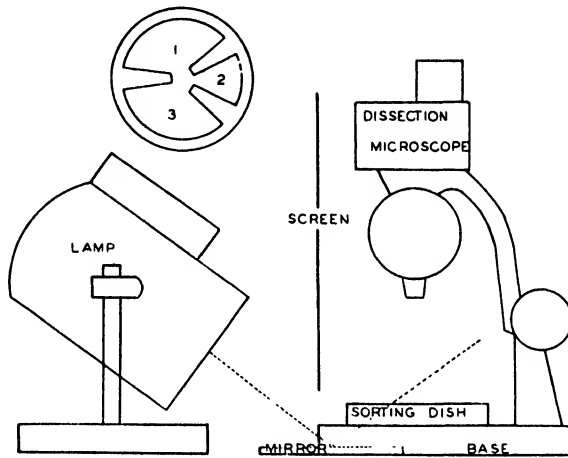


FIG. 1.—Diagram of setup used in examining eggs. Inset shows top view of sorting dish with its three compartments for unsorted eggs, discards, and satisfactory individuals.

The eyes during the diapause period and for the first 3 days of postdiapause development lie near the micropyle end of the egg. On the seventh day at 25° C., however, they are considerably increased in size and have moved to the anterior end (Slifer, 1932). It is thus possible by examination to select for use eggs which contain embryos and to note if growth occurs after treatment. The effects of experimental conditions on the breaking of diapause were made by observing development in this manner.

Eggs used in the experiments reported here, after being removed from the pods, washed, and the obviously bad ones discarded, were counted into lots of 100 and stored in castor dishes on damp filter paper. Each group was examined under the binocular dissecting microscope to be certain that every egg contained an embryo. All the dishes were kept continuously at 25° C. except those of the three experiments in which the effects of other temperatures were studied. At 7 days from the start of each determination the control eggs were examined to see how many had begun to develop. The experimental lots were counted similarly a week after the end of the treatment period. To observe the effect of centrifugal force, eggs were centrifuged in glass tubes under water for 20 minutes, at top speed in a clinical centrifuge (660 × gravity). Exposures to tem-

peratures of 0° , 10° , and 36° C. were carried out in refrigerators and in an electric oven, respectively. In the oxygen, nitrogen, and carbon dioxide experiments a sealed desiccator vessel containing dishes of eggs was filled with the gas. Eggs kept under water remained in their castor dishes with $\frac{1}{2}$ cm. of liquid covering them. Mild dehydration was effected by leaving eggs on dry filter paper. For more intense drying action they were kept on paper saturated with a ten-times isotonic sodium chloride solution. Treatment by all these methods (except centrifuging) was for a 6-day period, and at the close of this time the eggs were returned to the same conditions as the control groups.

RESULTS AND DISCUSSION

It is evident from the results presented in Figure 2 that there is a definite age difference in regard to susceptibility to the breaking of diapause. Those eggs that have just

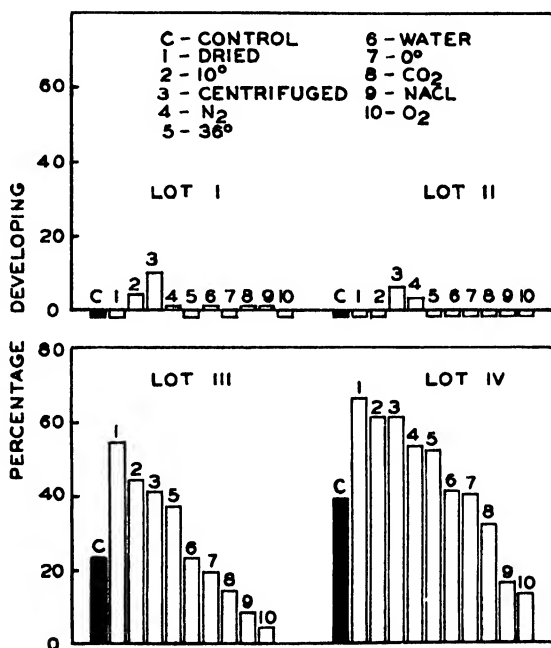


FIG. 2.—Graphs showing the effects of various treatments on breaking the diapause block in grasshopper eggs (for details see text). Ordinates represent the percentage developing within 7 days after end of treatment. At the start of the experiment the ages of lots 1-4, respectively, were 21, 39, 153, and 158 days; areas below the abscissa in lots 1 and 2 represent zero effect.

entered diapause and the ones that have been in this resting period for less than 3 weeks are almost entirely unaffected by any of the experimental treatments. The two lots that have been in diapause for about 20 weeks show much less ability to maintain themselves in this stage, and controls as well as experimentals have a greater tendency to develop. That this age effect is consistent and is not due to a peculiarity in choosing the samples is shown in Table 1, which represents results of centrifugation studies on 73 different lots of eggs with ages varying from 20 to 200 days.

A definite difference in the behavior of control eggs and centrifuged experimentals appears as early as the fourth week of diapause, and, as the age increases, more and more individuals leave the diapause state after being treated. By this procedure it is possible to estimate whether a given sample of eggs has been in diapause for a relatively long or short time. A more complete study of the influence of centrifuging on diapause is in preparation and will be published later. Table 1, however, is based on observations of over 7,000 eggs.

The centrifuging treatment was the only one used which had any consistent effect on the younger groups of eggs (Fig. 2); the following presentation will therefore concern itself only with the two older age groups shown.

Exposure to 0° C. for 6 days apparently had no effect on promoting development (Fig. 2). About the same number of individuals started postdiapause growth on removal as in the control lots. Ten degrees and 36° C. did tend to stimulate; about 60 per cent more eggs started to develop after these treatments than in the controls. Burdick (1937) states

TABLE 1*
DATA FROM CENTRIFUGING EXPERIMENTS ON SEVENTY-THREE
DIFFERENT GROUPS OF EGGS

AGE	AV. NO. DEVEL.		RANGE	
	Contr.	Exper.	Contr.	Exper.
20-40	0	2	0	0-10
40-60	0	9	0	0-32
60-80	0	23	0	1-51
160-180	17	32	9-25	13-48
180-200	26	43	16-49	30-60

* For details see text.

that short exposures to 10° C. have a negligible effect in reducing hatching time when the eggs are replaced at 28° C. The samples he used, however, had been kept for only 21 days at 28° C. previous to the low-temperature treatment and are therefore comparable with lots 1 and 2 in Figure 2. Burkholder (1934) observed that breaking diapause by heat appeared to injure the eggs, but the writers found that the ones kept at 36° C. in the present series were apparently not harmfully affected and hatched normally.

The effect of storing the eggs in an atmosphere of oxygen for 6 days seems to be inhibitory. In the 158-day lot only one-third as many eggs began development within the 2-week period following the start of the exposure to the gas as in the control group. In the 153-day group the proportion was still less, approximately one-sixth. Figure 3, A, showing experiments on 11 lots of eggs of different ages, indicates the same result, that is, delay in the start of development after exposure to high oxygen tension.

Decreasing the metabolic rate through depriving the eggs of oxygen by keeping them in an atmosphere of nitrogen has the opposite effect. Thirty-six per cent more embryos started growth after this treatment than in the controls (Fig. 2, lot 4). Figure 3, B, shows a more complete age series of similar experiments with qualitatively the same result.

Lowering the oxygen tension by keeping the eggs covered with water seems to have no effect, at least during the 6-day interval tried (Fig. 2). The dissolved oxygen in the water may partially account for the difference between this lot and the ones kept in nitrogen.

It was thought that the breaking of diapause by low temperatures might be partly effected by the "piling-up" of carbon dioxide in the covered storage dishes. The action of a carbon dioxide atmosphere, however, seems to be retarding rather than stimulating (Fig. 2).

Disturbance of the water balance within the egg system has a pronounced effect on the initiation of development. Leaving the eggs in air on dry filter paper, although it had no influence on the two younger lots shown in Figure 2, changed markedly the number developing in the two older groups. One of these showed a 69 per cent increase over the controls, and the other 134 per cent. Although the eggs do not lose their shape in this mild dehydration, they take up water rapidly when the paper on which they rest is moistened, and it is perhaps this process that is significant. Schipper (1938) noted that in some cases "dehydration-rehydration may serve to shorten or eliminate the developmental block."

In the more vigorous dehydration obtained by placing eggs on filter paper wet with a ten-times isotonic sodium chloride solution enough water was removed to cause the eggs to lose considerable weight and some of them to appear shrunken. In this case some injury may have occurred, as all the eggs did not regain their original volume within a few days after being washed and replaced on filter paper moistened with distilled water. The treatment caused a definite decrease in the percentage of eggs which later developed, second only to that produced by the high oxygen tension and amounting to 51 per cent and 65 per cent of the controls in the two groups reported (Fig. 2).

From the results presented, it appears evident that the diapause condition decreases in stability as the period proceeds and that during its later stages abnormal treatment of various sorts may cause the eggs to resume active development. It is apparent also that during the earlier part of the period the egg is in a state quite resistant to any disturbing agent, and no method yet tried is even 25 per cent effective in breaking the block.

For purposes of speculation the five methods tried, which successfully break diapause in some of the older eggs, may be divided into two groups, the ones which may affect water balance and those that lower the metabolism. An increase in the water content of the egg seems to be associated with morphological development. It has been reported that the percentage of water in the whole egg becomes higher as prediapause and post-

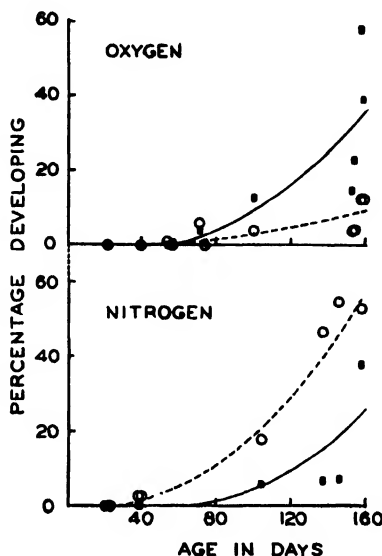


FIG. 3.—The action of atmospheres of oxygen and nitrogen on breaking diapause in grasshopper eggs (for details of exposures see text). Abscissae represent age of the eggs in days from laying; ordinates, percentage developing within a week after end of exposure; closed squares, controls; open circles, experimentals.

diapause growth proceeds (Bodine, 1929; Slifer, 1938). Unreported observations of the writers also indicate that with increasing age in diapause and the accompanying decrease in stability, there is water uptake by the egg. It may be, then, that a change which brings about intake of water results in the breaking of diapause. Drying, centrifuging, and exposure to 36° C. may all lead to an absorption of water by the egg, but it is difficult to determine this accurately by weighing. In a group of eggs so treated some individuals will develop and, in doing so, take up water and increase in weight. Since it is impracticable to distinguish morphologically between these eggs and those still in diapause during the first 2 days of postdiapause development, they cannot be separated from the group, and their weight increase makes the gross weight of the group unrepresentative for the matter in question. Measurements of the weights of single eggs, whose development could then be followed, is also impracticable, because the degree of accuracy of the method overlaps the range of variation in the magnitudes being compared. Determination of the densities of individual eggs by a flotation method is being done at present and may give evidence bearing on this question.

Effects of a low level of metabolism are apparent in results of the experiments at a temperature of 10° C. and also in an atmosphere of nitrogen (Fig. 2). A question arises as to the lack of effect of 0° compared with 10° C. With an increased exposure at the former temperature, diapause is successfully broken; whether 10° C. is the more effective over a long period in destroying the block is yet undetermined.

Structure may possibly be another factor concerned in the potency of the block. Stuart (1935) reports that drying causes extensive disintegration of yolk cells within the egg. Centrifuging undoubtedly has a similar effect. If this action liberates from these cells a substance necessary for resumption of growth or destroys a factor that has been inhibiting development, the observed effects might occur. However, this conception is purely hypothetical.

More extensive investigations are under way at present in an attempt to understand more clearly the factors responsible for the maintenance and destruction of the diapause state.

SUMMARY

1. The stability of the diapause state in the egg of the grasshopper, *M. differentialis*, has been studied by subjecting eggs to various experimental conditions, including centrifugation, drying, temperatures of 10° and 36° C., and atmospheres of nitrogen, oxygen, and carbon dioxide. The number of individuals starting active development after the treatment was determined.
2. The stability of the diapause state varies with the age of the eggs, the younger ones being relatively resistant to all the treatments used, while the older ones are more easily induced to resume growth.
3. Treatments which were most effective in breaking diapause in old eggs are drying and wetting, centrifugation, exposure to nitrogen, and temperatures of 10° and 36° C.
4. Exposure to oxygen, carbon dioxide, and hypertonic sodium chloride solutions resulted in a smaller number of eggs breaking diapause than in the control groups.
5. A discussion is given of the possible correlation of the egg behavior with water balance, level of metabolism, or cellular structure.

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RESPIRATION DURING FLIGHT IN DROSOPHILA REPLETA WOLLASTON: THE OXYGEN CONSUMPTION CONSID- ERED IN RELATION TO THE WING-RATE

(Three figures)

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THE object of this study has been to obtain new information concerning the respiratory metabolism of an insect during flight. No previous paper known to the present writers treats quantitatively the respiration during flight of any dipterous insect, although Demoll (1927) inferred that the rate of respiration of mosquitoes (*Culex* sp.) must be increased in flight because they were then more rapidly

TABLE 1
DATA ON FLIGHT RESPIRATION RECORDED IN THE LITERATURE

Author	Species	Temper- ature (° C.)	O ₂ Up- take at Rest (Cu. Mm. per Gram per Min- ute)	O ₂ Up- take in Flight (Cu. Mm. per Gram per Min- ute)	O ₂ Uptake in Flight O ₂ Uptake at Rest	Remarks
Tauchert . . .	<i>Apis mellifica</i>	21	1,217	...	Running about very ac- tively; sometimes flying
Kosmin, Alpa- tov, and Res- nitschenko . .	<i>A. mellifica</i>	{ 35 18 11	{ 9 4	{ 4,600 5,200 4,400	{ 580 1,100
Jongbloed and Wiersma	<i>A. mellifica</i>	{ 21 21	{ 32 33	{ 1,670 1,274	{ 52 39	Good flyers Poor flyers
Kalmus	<i>Deilephila el- penor</i>	20-22	2.8	104	37
Raffy and Portier	{ <i>Thais cassan- dra</i> <i>Sphinx ligustri</i>	{ 20-25 20-25	{ 6.7 13.3	{ 3,605 400	{ 540 30	Fluttering of wings in- duced by nicotine poi- soning

poisoned by HCN than when at rest. Data on the respiration of flying bees (*Apis mellifica* L.) are given in the papers of Tauchert (1930), of Kosmin, Alpatov, and Resnitschenko (1932), and of Jongbloed and Wiersma (1934), while Kalmus (1929), and Raffy and Portier (1931) have studied the respiration of flying Lepidoptera. The literature has been reviewed in some detail by Jongbloed and Wiersma, so that it will be necessary to give here only a brief summary of the results obtained. This has been done in Table 1.

¹ James King of Irrawang Travelling Scholar of the University of Sydney.

In addition to these, mention should be made of the works of Parhon (1909) and Steidle-Zander (1921), who measured the oxygen uptake of swarms of bees, in which the insects, although probably not all flying, must have been in a state of considerable activity. Parhon obtained a value of 289 cu. mm. per gram per minute at 20° C., while Steidle-Zander reported at 20°–25° C. an oxygen uptake of only 34 cu. mm. per gram per minute.

Some of the evident disagreements in the literature are due to the wide range of variation which exists in the rate of respiration of different specimens, even of the same species; others are due to the different methods of measurement used by the various workers; and still others arise from the difficulty of estimating accurately the degree of activity of the specimens during the period of measurement. In the present study the rate of respiration has been recorded continuously, using a manometric method, while the rate of wing beat has been followed simultaneously with a stroboscope. It has been possible thus to make a direct comparison of the rate of respiration and the degree of activity and in this way to account for some of the variation in the rate of respiration of a single individual during flight.

The correlation of wing-rate and rate of oxygen consumption also allows a more accurate estimate of the energy requirements of the active flight muscles than could be made on the basis of past determinations. Such measurements are of particular interest, since the flight muscles of insects so greatly surpass most other contractile tissues in their capacity for repeated response at a rapid rate. In the species studied here the rate of wing beat is regularly of the order of 100–250 strokes per second, and fastened specimens have flown for periods of as much as 40 minutes, in the course of which the flight muscles will have performed several hundred thousand successive contractions.

METHODS

Specimens of *Drosophila repleta* Wollaston were selected at random from the same self-maintained culture that had been used in previous studies on wing-rate (Chadwick, 1939).

Oxygen uptake was measured in Warburg manometers having small cylindrical vessels of about 2.5 cu. cm. capacity. The insects were fastened in the center of the vessel and made to fly by the sudden removal from under the tarsi of a supporting platform. This was effected by means of a springboard arrangement, which was operated by an electromagnet situated outside the vessel.

The flies were mounted in the manner shown in Figure 1, being held by means of a small slip of paper attached with paraffin to the tip of the abdomen. The spring consisted of a small, flexible bristle (1), attached above to a small piece of glass (2), which was also provided with a projection (3) designed to carry the slip of paper supporting the insect. The platform (4) was of blackened paper, 6×4 mm., with a piece of iron wire fixed to the underside. The glass base (2) was fastened firmly by means of paraffin wax to the plug of the manometer at one side of the central bore. The variation in volume of the paper mounts for the insects and the amount of paraffin used in attaching them was small enough that it was not necessary to redetermine the volume of mount and spring for each specimen.

To insure rapid absorption of carbon dioxide, 0.06 cu. cm. of 10 per cent KOH was placed in the bottom of the vessel, as well as 0.04 cu. cm. in the side arm. All experiments were run in a water bath at a temperature of 25° C. The electric stroboscope was

placed directly above the manometer vessel. The insects were observed in silhouette through the bottom of the vessel, with the aid of a glass panel in the bath and a system of magnifying glasses and mirrors. The magnet was placed in a glass jar immersed in the

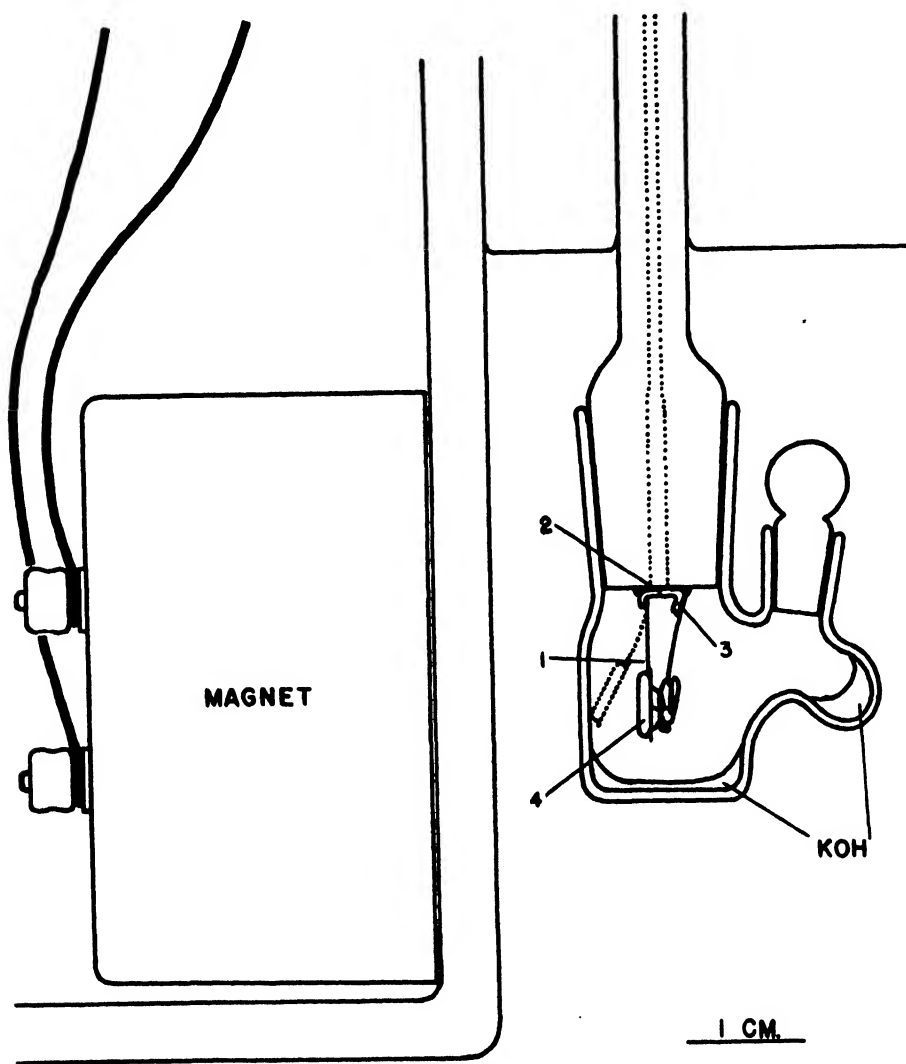


FIG. 1.—Apparatus used in studying the respiration during flight in *Drosophila*

water bath and arranged at a distance of not less than $\frac{1}{4}$ cm. from the experimental chamber. An adequate circulation was maintained in the bath, so that there was a constant streaming of water between the two vessels. During flight, readings of wing-rate were made every 10 seconds, and manometer readings every 30 seconds. Under the

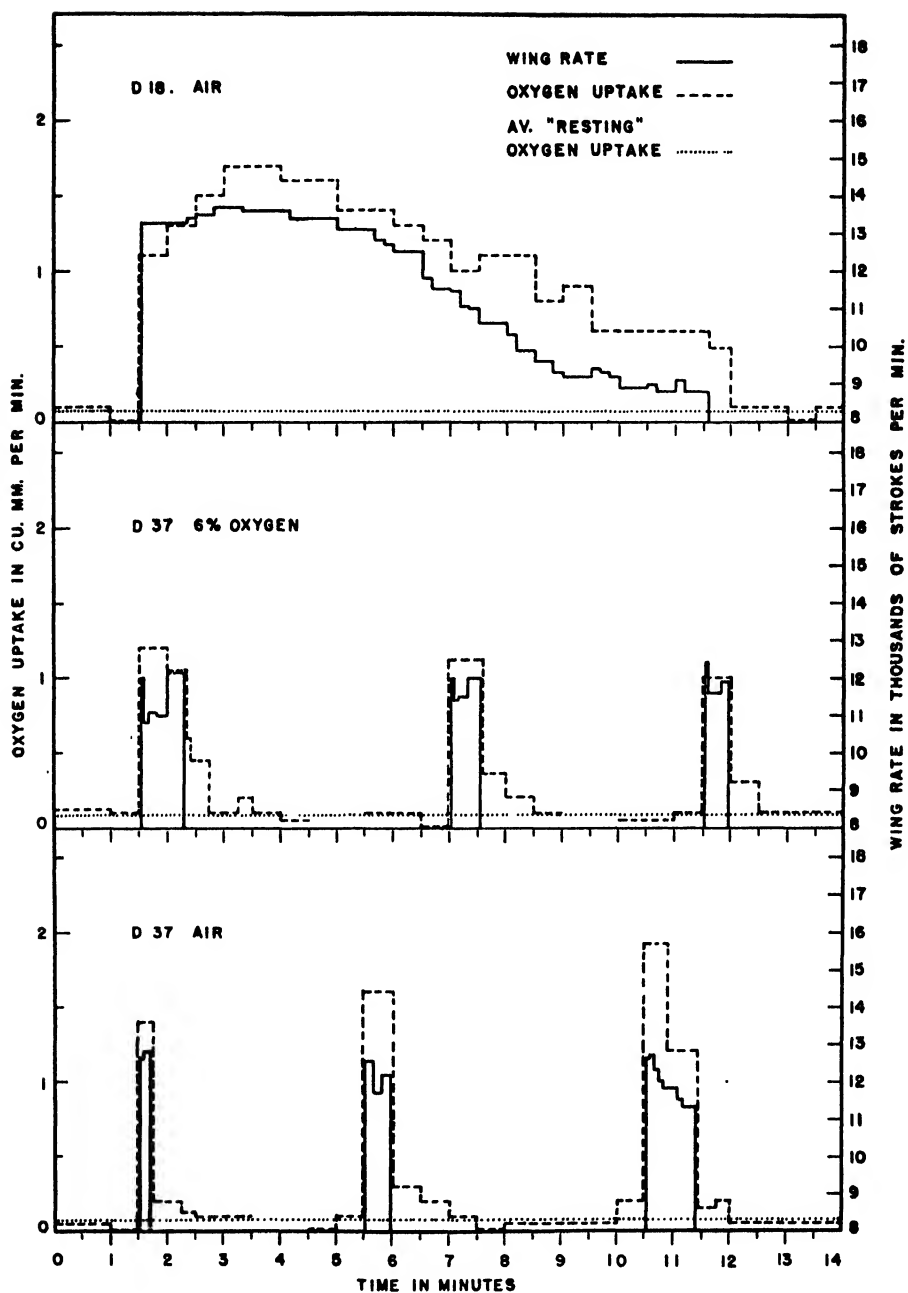


FIG. 2.—Rates of oxygen consumption and wing movement during individual flights in *D. repleta* Wollaston. The resting level of oxygen consumption is an average of all the readings taken during resting periods on each insect.

conditions described, manometer excursions of from 1 to 5.5 mm. per half-minute, depending on the size and wing-rate of the animal, were obtained. The intensity of the "resting" metabolism was followed between flights and at the beginning and end of each experiment. Except in one instance, the insects were allowed to fly until they stopped of their own accord. In a series of blank controls it was determined that there was no change in pressure in the vessel as a result of running either the stroboscope or the magnet.

RESULTS AND DISCUSSIONS

MAGNITUDE OF THE GASEOUS EXCHANGES

Data were obtained on 16 insects which made a total of 76 flights ranging in duration from 15 seconds to 40 minutes 45 seconds. The results of several of these flights are presented graphically in Figure 2.

TABLE 2
FLIGHTS OF MORE THAN 1 MINUTE IN LENGTH*

SPECIMEN	WEIGHT (MG.)	O ₂ UPTAKE AT REST (Cu. Mm. PER GRAM PER MINUTE)	TOTAL FLYING TIME (MIN.:SEC.)	AVERAGE WING-RATE (STROKES PER MINUTE)	O ₂ UPTAKE DURING FLIGHT		O ₂ UPTAKE IN FLIGHT
					Cu. Mm. per Minute	Cu. Mm. per Gram per Minute	
D3 ♀	2 4	20 5	3:50	10,310	0 77	337	11
D4 ♀	3 0	24 8	12:15	10,240	1 32	351	14
D9 ♀	5 2	25 5	12:10	12,770	1 75	349	14
D10 ♀	3 2	30.1	15:25	13,080	1.43	462	15
D13 ♀	4 3	20 0	7:20	12,270	1 66	419	14
D17 ♀	4 2	20 7	21:05	10,030	0.95	242	8
D18 ♀	2 9	23 6	11:30	11,930	1.12	399	17
D20 ♂	1 4	24 1	2:45	10,040	0 52	383	16
D21 ♀	2 1	23 0	32:20	9,540	0 78	382	17
D26 ♂	1 6	32 7	37:55	8,420	0 41	273	8
D31 ♀ (6 per cent O ₂)	4 6	22 7	1:40	10,490	1 00	230	10
D31 ♀	4 6	22 7	55:35	10,020	1 11	256	11
D37 ♀ (6 per cent O ₂)	4 1	20 6	3:20	11,820	1.20	302	15
D37 ♀	4 1	20 6	7:50	11,030	1 51	379	18
D38 ♀	2 8	45 7	9:55	11,880	1 33	499	11
Av.		27 8		10,920	1.12	351	13

* Insects flown in air unless otherwise stated.

The intensity of respiration at rest and during flight for the various animals is shown in Table 2. The average oxygen uptake is 28 cu. mm. per gram per minute during rest and 350 cu. mm. per gram per minute during flight, the increase being 13 times. During these flights the wing-rate averaged 10,920 strokes per minute. Both oxygen uptake and wing-rate may vary widely on either side of the average values. The greatest increase in oxygen uptake observed during flight was 26 times the resting value (D37, flying at a rate of 12,920 strokes per minute); the least, 5 times (D26, flying at a rate of 7,200 strokes per minute).

A number of flights were made in vessels containing only a small amount of water instead of KOH. In these experiments no significant change was noticed in the manometer reading, either during or after flight. The respiratory quotient thus appears to be 1, and carbon dioxide is given off by the insect as soon as oxygen uptake commences.

The average weight of the thoracic muscles is approximately 0.6 mgm., as determined by subtracting the dry weight of the thoracic skeleton after treatment with KOH from the weight of the freshly killed thorax.

From these data a number of calculations can be made, as follows: The average oxygen uptake per wing stroke, i.e., per single twitch (since each complete stroke represents a single contraction of each of two separate sets of muscles), is approximately 2×10^{-4} cu. cm. per gram of muscle. This represents the oxidation of 2.5×10^{-7} gm. glycogen and the liberation of 1×10^{-3} calorie of energy per gram of muscle for each contraction.

It is instructive to compare these figures with data obtained for frog muscle under somewhat similar conditions. Hill and Kupalov (1929) have obtained the following figures for an isolated muscle contracting isometrically in oxygenated Ringer's solution, the frequency of stimulation being such that there was no accumulation of lactic acid.

Average oxygen consumption per	
single twitch	8×10^{-4} cu. cm. per gram muscle
Glycogen oxidized per single twitch . .	11×10^{-7} gm. per gram muscle
Total heat production per single	
twitch	4×10^{-3} cal. per gram muscle

The average energy output per minute during flight in *D. repleta* is 11 cal. per gram muscle, at an average rate of contraction of 10,920 strokes per minute. In frog muscle contracting 23 times per minute the energy liberated each minute is 9×10^{-2} cal. per gram, and at the theoretically maximum rate of contraction of 37 times per minute in a "steady state" (Hill and Kupalov) it would be 15×10^{-2} cal. per gram.

A fly of average weight 3.5 mg. burning glycogen at the rate given above would use 0.1 mg. per hour of flight, i.e., 3 per cent of the body weight, or about 15 per cent of the weight of the wing muscles. Long flights must thus produce a considerable exhaustion of the total carbohydrate supplies of the body. It is possible that the amount of sugar in the blood is very high, as it is in the honeybee, in which Beutler (1936) found average blood sugar values of 2 per cent. According to Beutler, the total carbohydrate of bees of 100 mg. weight averaged 2.6 mg., and this supported 15 minutes of flight, during which the consumption of glucose was 8.5 mg. per hour. Jongbloed and Wiersma (1934) demonstrated a glucose consumption during flight of about 10 mg. per hour per bee. Given a total carbohydrate reserve in *Drosophila* similar to that in the bee, the total possible flying time should be rather less than 1 hour in a fly of 3.5 mg. weight. This agrees fairly well with the maximum flying times obtained in our experiments (Table 2: D21, D26, D31). The flights during which the respiratory quotient was followed were of relatively short duration (6 and 5 minutes), so we are unable to state whether or not substances other than carbohydrates act as sources of energy as the point of exhaustion is approached.

OXYGEN DEBT

It is to be noted that the oxygen uptake rises sharply at the beginning of flight and falls off rapidly immediately activity ceases (Fig. 2). There is a slight lag, however,

particularly noticeable at the end of flight, where the oxygen uptake remains considerably above the resting value for a period of usually 1-2 minutes after flight ceases. An explanation of this lag might be sought in any or all of the following factors: (1) lag in the apparatus, due to the time interval between production and absorption of carbon dioxide; (2) pressure changes in the vessel, due to heat production by the insect; (3) recovery processes in the muscles.

In order to test the first of these possibilities, a sleeve of Whatman filter paper, fitting around the inside of the vessel, was introduced in one series of flights. In spite of the great increase in absorbing surface of KOH provided in this way, no effect whatever on the magnitude or duration of the delayed oxygen uptake was observed. It seems, therefore, that whatever lag there is in the apparatus cannot be of much significance. This is not surprising, in view of the very efficient mixing of air accomplished by the wings of the flying insect.

That heat production by the insect is not responsible is shown by the fact that no change in the manometer reading was recorded in the experiments in which the KOH was replaced by water and the carbon dioxide not absorbed.

It is probable, therefore, that the continued high rate of oxygen uptake after flight ceases is due largely to the removal of lactic acid or some comparable substance accumulated during flight and, to a certain extent, to the restoration to the normal value of the oxygen tension of the tissues. During activity this must necessarily have fallen to near zero in order to maintain a maximum rate of entry of oxygen in a system which depends, at least in part, on gaseous diffusion. That we are concerned here with a true oxygen debt is further demonstrated by the fact that, in short flights of different durations, the ratio of the oxygen consumed in excess of resting requirements to the number of wing strokes is much more constant when the total excess of oxygen uptake (both during and after flight) is considered rather than the excess during only the actual flying time.

Table 3 shows the magnitude of the oxygen debt in a number of specimens. The average value from all flights is 0.18 cu. mm. The debt seems to be relatively constant for any one insect, regardless of the duration of flight, and is not obviously related to the body weight.

In experiments 31 and 37 the effect of a relative deficiency of oxygen supply on the course of oxygen uptake during flight was tested by flying the insects in a mixture of approximately 6 per cent oxygen in nitrogen. Difficulty was always encountered in inducing the insects to fly in this mixture, and the flights were invariably short (longest, 3 minutes, 20 seconds) and at a slower rate than was characteristic of the same animal in air. (It should be noted that this does not appear in Table 2, since the flights in air ranged over a wider series of rates and averaged lower than did the short flights in the 6 per cent mixture. The initial rates in air were always higher than those in 6 per cent oxygen.) The oxygen debt in these experiments did not differ markedly from that observed when the same individuals were flown in air.

The average value for the oxygen debt of 0.18 cu. mm. represents the amount of oxygen that would be used in making 1,000 contractions, corresponding to an accumulation of lactic acid in the muscles of about 0.2 per cent (assuming an oxidative quotient similar to that in frog muscle). In the experiments of Hill and Kupalov (*loc. cit.*) an isolated frog muscle was able to make, on the average, 700 contractions in nitrogen, accumulating lactic acid to a concentration of 0.3 per cent.

Whether or not the termination of flight is due to the fact that the concentration of lactic acid has reached a certain saturation value is not certain, although the relative constancy of the oxygen debt in different flights of a single individual points to this conclusion. But there certainly can be no constant rate of accumulation of lactic acid during flight in air, since the magnitude of the oxygen debt is independent of the length of

TABLE 3
MAGNITUDE OF THE OXYGEN DEBT*

Specimen	Length of Flight (Min.:Sec.)	Oxygen Debt (Cu. Mm.)	Specimen	Length of Flight (Min.:Sec.)	Oxygen Debt (Cu. Mm.)
D9 ♀	0:40	0.23	D31 ♀ (6 per cent O ₂ continued) ..	0:15	0.17
	1:10	0.29		0:15	0.16
	1:20	0.17		0:15	0.21
	0:55	0.17		0:20	0.13
	0:50	0.17			
	0:15	0.08	Av.		0.19
Av.		0.19	D31 ♀ (air) ..	40:45	0.23
D13 ♀	1:35	0.54		8:50	0.10
	1:25	0.38		6:00	0.11
	2:00	0.60	Av.		0.15
	2:20	0.45			
Av.		0.52	D37 ♀ (6 per cent O ₂)	0:50	0.17
D17 ♀	0:45	0.26		3:20	0.04
	0:35	0.03		0:35	0.18
	5:10	0.15		0:20	0.00
	3:45	0.03		0:57	0.06
	2:25	0.04		0:30	0.11
	0:30	0.10		0:15	0.04
				0:15	0.13
Av.		0.10	Av.		0.09
D31 ♀ (6 per cent O ₂)	1:40	0.17	D37 ♀ (air) ..	0:15	0.00
	0:35	0.20		0:15	0.10
	0:35	0.25		0:25	0.11
	0:30	0.15		0:30	0.17
	0:22	0.24		0:55	0.05
	0:28	0.20		0:25	0.15
	0:15	0.00		1:35	0.08
			Av.		0.09

* For weights see Table 2. Insects flown in air unless otherwise stated

flight. Limitation of the duration of flight in air may perhaps be explained more readily by the disappearance of some essential substance, such as carbohydrate. Certainly, the total possible flying time depends on the amount of reserve foodstuffs present, and it is possible that the termination of individual short flights is brought about by a temporary lack of substrate.

In the low-percentage oxygen mixture both length of flight and wing-rate are reduced, so that oxygen supply does seem to be a limiting factor. This is especially evident in the case of specimen D31, which, after 11 flights in 6 per cent oxygen, the longest of which

lasted only 1 minute 40 seconds, flew for successive periods of 40, 8, and 6 minutes in air. One would expect that for flights in 6 per cent oxygen the oxygen debt would be an expression of a maximal concentration of lactic acid in the muscles.

Failure to detect any difference in the magnitude of the oxygen debt between the flights in air and those in the 6 per cent oxygen mixture may be due to insufficient sensitivity of the apparatus, in view of the very small quantities involved, but it is also possible that the oxygen debt is an expression of a condition of exhaustion induced by two different means—in air by the irremediable disappearance of some substance connected with the oxidation process and in 6 per cent oxygen by the actual insufficiency of the oxygen supply.

OXYGEN UPTAKE IN RELATION TO WING-RATE

The wing movement may be considered theoretically to approximate a sine curve. Actually, up- and downstrokes are not quite symmetrical, and the retardation at the end of the stroke is rather greater than in a simple harmonic motion, but the experimental results may be expected to agree fairly well with a theoretical equation derived on this basis.

In a motion of this type the velocity at any point is equal to $v_0 \sin \omega t$, where v_0 is the maximum velocity at zero phase and ωt is the angle swept out in time t at an angular velocity ω .

Since the resistance to a body such as an insect's wing moving in air is proportional to the square of the velocity, over the range of velocities encountered, the work W done in any stroke should be proportional to the square of the average velocity of the wings (provided the angle of the wings in relation to their direction of movement does not vary from stroke to stroke), i.e.,

$$\begin{aligned} W &= K v_0^2 \int_0^{2\pi} \sin^2 \omega t \cdot d\omega t, \\ &= K v_0^2 \pi, \\ &= K' v_0^2. \end{aligned}$$

But

$$v_0 = \omega \times \text{amplitude } (a),$$

and

$$\omega = 2\pi \times \text{frequency } (f);$$

therefore

$$v_0 = 2\pi a f,$$

and

$$W = K'' a^2 f^2,$$

or for any given animal, since the amplitude is apparently constant,

$$W = K''' f^2.$$

That is, the oxygen uptake per stroke should be proportional to the square of the wing-rate.

The experimental data are set out in Table 4, and, in Figure 3, f^2 has been plotted against the oxygen uptake per stroke for the three insects which flew the longest times. In the case of D26 and D31 the points lie along straight lines passing through the origin.

TABLE 4
OXYGEN UPTAKE PER STROKE IN RELATION TO WING-RATE*

Specimen	Number of Observations	Frequency (f) (Strokes per Min.)	(f) ² × 10 ⁻⁶	Oxygen Uptake per Stroke (Cu. Mm. × 10 ⁶)	(f) ²
					Oxygen Uptake per Stroke (× 10 ⁻¹⁰)
D4.....	7	11,330	128	131	98
	10	10,400	108	130	83
	3	9,510	90	105	86
	4	8,680	76	104	73
D9.....	10	13,350	170	130	129
	14	12,530	157	127	124
	4	11,620	135	105	129
D17.....	4	12,270	151	113	134
	14	11,470	132	88	150
	9	10,680	113	78	145
	5	9,550	92	92	100
	2	8,430	73	80	82
	6	7,440	55	70	70
	2	6,550	42	90	42
D18.....	8	13,460	181	106	171
	3	12,600	161	90	170
	4	11,470	132	97	136
	2	10,310	108	101	107
	3	9,330	87	75	116
	4	8,880	79	60	132
D21...	12	11,270	127	91	140
	13	10,270	105	82	128
	19	9,530	91	77	118
	9	8,510	72	68	106
	10	7,710	59	62	95
D26.....	22	9,380	88	56	152
	33	8,400	71	45	158
	23	7,590	58	35	166
D31.....	4	12,190	140	147	101
	14	11,440	131	125	105
	30	10,310	106	112	95
	48	9,540	93	97	96
	10	8,800	77	85	91
D37.....	6	12,640	160	151	106
	2	11,650	136	140	97
	2	10,550	112	135	83
	2	9,670	94	111	85
	5	8,640	75	92	82
D38.....	11	12,470	155	117	132
	7	11,510	133	102	130
	3	10,770	116	78	149

* Results averaged in intervals of 1,000 wing strokes per minute. Included are all insects which flew for more than 7 minutes, with the exception of D10 and D13, in which the range of wing-rates was very small. In the figures for oxygen uptake per stroke only the oxygen consumed in excess of the resting requirements is considered.

Number D21, however, does not agree with the theoretical equation. Of the insects listed in Table 4, D9, D26, D31, and D38 show a fairly close agreement with the expected relation; D37 agrees over part, at least, of its range of frequencies; while in D4 and D18 the scatter is rather too great to warrant a definite decision. Numbers D17 and D21 cannot be made to fit straight lines passing through the origin, and of these D17 shows so much scatter as to suggest that the points are supported by too few observations to be of much significance.

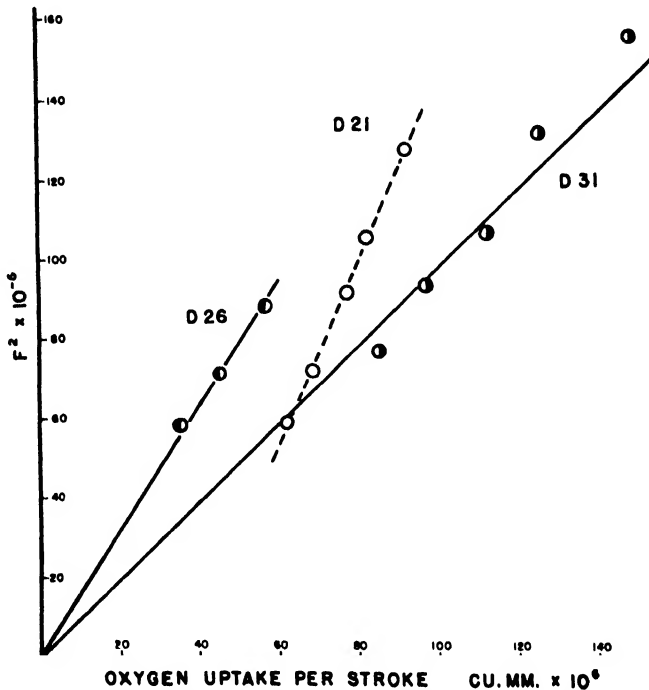


FIG. 3.—Relationship between the oxygen uptake per wing-stroke and the square of the wing-rate different specimens of *D. repleta* Wollaston.

We were not able to measure the amplitude during the course of flight, and, although there were no obvious changes, minor variation may have occurred in some cases. Since this factor enters the equation as the square, small variations would be magnified in their effect on the oxygen uptake per stroke. It is also possible that the pitch of the wings, and hence the mass of air moved per stroke, may be altered at some time during flight. Variations of this nature may have obscured the true relation between oxygen uptake per stroke and wing-rate in insects such as D21. The fact that the majority of the specimens, including those in which the figures are supported by the greatest number of observations, do agree with the theoretical relation suggests that the oxygen uptake per stroke is, in fact, proportional to the square of the wing-rate.

The constant K''' , besides involving the amplitude of the stroke, must also include a number of factors depending on such quantities as body weight, area of wings, etc. It is

hardly to be expected that a constant relation between the one known factor among these—i.e., the body weight—and the oxygen uptake during flight could be demonstrated. And, in fact, when the body weight of the different individuals is plotted against average oxygen uptake per stroke *divided by* (average wing rate)², no relation does appear.

COMPARISON WITH PREVIOUS WORK

The percentage increase in respiration during flight that we have reported is considerably lower than the values previously obtained for flying insects. This may be due in part to the fact that the energy required for flight should be relatively less in a smaller insect, where the surface/mass ratio is larger. Although lower, our results are of the same order of magnitude as those of Jongbloed and Wiersma and those of Kalmus and the lower values reported by Raffy and Portier. It is to be noted that the increase of 540 times reported for one specimen by Raffy and Portier was obtained from an insect which "flew" for only 1 minute.

The average values which have been reported for respiration in bees, both at rest and in flight, are considerably higher than in other insects. The values obtained by Kalmus for *Deilephila* (Lepidoptera), on the other hand, are relatively very low. It is possible that in his experiments there was some loss of carbon dioxide in the paraffin oil over which the gases to be analyzed were collected.

The results of Kosmin *et al.* show much greater increases during flight than any others reported. These authors, however, seem to have used only one bee at each of the temperatures at which experiments were run, and the fact that, beyond stating that the insects were flown in Barcroft manometer vessels, they give no details of their methods makes the significance of their results difficult to assess. They state as a prerequisite for measuring the resting metabolism that "the animal must first be starved for 5-10 hours at 10°-11° C., until it is beginning to become stiff." It is not surprising that the resting values obtained under such conditions are very low.

Of course, no very rigid comparisons can be made when the figures under discussion depend on a purely arbitrary "resting respiration." In our experiments this value was measured with the animals held in a fixed position. Under these conditions they usually remained quiet, but there was a tendency to struggle at times. The results agree very well, however, with those of 2 experiments in which the flies were not fastened and the respiration was followed in darkness—conditions under which the insects remain without movement for long periods of time. The average of 17 experiments, ranging in duration from 20 to 200 minutes (i.e., excluding some of the values based on shorter experiments included in Table 1) was 24 cu. mm. per gram per minute, or 1,440 cu. mm. per gram per hour. Values obtained by Kucera (1934) for *D. melanogaster* Meigen at 26° C. were 1,660 cu. mm. per gram per hour for males and 2,380 cu. mm. per gram per hour for females.

SUMMARY

The average oxygen uptake found for *D. repleta* was 28 cu. mm. per gram per minute during rest and 350 cu. mm. per gram per minute during flight (average of flights of over 1 minute in duration). This represents an increase during flight of 13 times the resting value. The average wing-rate during the same flights was 10,920 strokes per minute.

The average oxygen consumption per wing stroke was approximately 2×10^{-4} cu. cm.

per gram of muscle, representing the oxidation of 2.5×10^{-7} gm. glycogen, the respiratory quotient being 1, and the liberation of 1×10^{-3} cal. of energy per gram of muscle.

Although there is some disagreement in the results, the data suggest that the relation between oxygen uptake per stroke and frequency of wing beat is such that the oxygen uptake per stroke is proportional to the square of the frequency.

The average oxygen debt, which was paid off during the first 2 minutes after the end of flight, was 0.18 cu. mm. This was not altered significantly when the same insects were flown in an atmosphere of 6 per cent oxygen in nitrogen, although both the duration of flight and the initial wing-rate were reduced in this mixture.

A comparison of our results with those reported previously indicates that the bulk of the evidence does not support the claim that extremely large increases in the rate of respiration occur during flight.

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THE RESPIRATORY METABOLISM OF THE FROG RETINA

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A REVIEW of the literature reveals a surprising lack of experimental work on the metabolism of the retina. For a number of reasons it would appear that the retina provides ideal material for studies on the metabolism of nervous tissue. In the first place, it consists primarily of nervous elements, originating embryologically from the brain; and, secondly, it can be readily removed from the eye with minimal injury to the cells and is thin enough to fall within the thickness generally required for respiratory studies, therefore obviating the necessity for slicing.

Among the earliest studies on the metabolism of the retina were those of Warburg and Nagelein (1929) and Nakashima (1929), who reported that light had no effect upon the rate of respiration in rats and fishes. Nakashima and Hayoski (1933) reported that the oxidation-reduction potential of suspensions or extracts of frog retina in Ringer's solution rises when the preparation is exposed to light and falls again when the light is removed.

Jongbloed and Noyens (1936) presented data in which they showed that the gaseous exchange in half-eyes of frogs was increased about 20 per cent in the dark. They attributed this increase in respiration to the resynthesis of visual purple. Chase and Smith (1939), however, reported that frog retina tested in light and dark showed no difference in the rate of oxygen consumption.

There seems to be no general agreement at the present time relative to the question of the effect of light on the gaseous exchange of the retina. Moreover, the work that has been done on the respiration of this tissue contributes relatively little to a general understanding of the complex chemical changes which undoubtedly take place under various conditions.

Considering the foregoing, it is obvious that the effects of dark and light should be further investigated before work can progress on the metabolism of the retina. Therefore, an attempt has been made, first, to study the rate of oxygen consumption in the frog's retina under controlled conditions of darkness and light and, secondly, to determine the amount of oxygen consumed per milligram dry weight of tissue under uniform illumination.

MATERIAL AND METHODS

The tissue used in this experiment was the retina of the frog, *Rana pipiens*. The frogs were obtained in groups of one hundred or more (approximately every three weeks) over a period of one year; and the data are presented with no regard to seasonal variation.

The retinas were prepared in the following manner: The frogs were decapitated, and their heads skinned; then the cornea and lens were removed. By means of curved forceps an approach was made into the eye between the choroid and the retina. A firm hold was obtained upon the optic nerve, and the retina was lifted from its position in the eye.

All experiments were performed with freshly prepared Ringer's solution brought to a

pH of 7.5 with Sorensen's phosphate buffer. The Ringer's solution was prepared each time from stock solutions of M/9 Baker analyzed grade of NaCl, KCl, and CaCl₂ in proportions of 100:1.5:1.

With the exception of a few experiments where a modified Fenn manometer was used, the data presented in this paper were obtained by the Warburg manometric method. The direct method described by Dixon for measuring oxygen consumption was used, and the manometers were filled with air. The rate of oxygen consumption in the series on the effect of light and darkness is expressed in millimeters change on the manometer index scale for comparable intervals of time, varying in different experiments from 5 to 15 minutes. In the second series the rate of oxygen consumption is expressed in cubic millimeters of oxygen consumed per milligram dry weight of tissue per hour. After each experiment the retinas were removed from the manometer flask and placed in weighing cells, dried for 10 hours at a temperature of 40° C., then weighed.

RESULTS AND DISCUSSION

Before pursuing the problem on the metabolism of the retina, it seemed desirable to establish whether the rate of oxygen consumption was different in darkness and in light. Data were obtained from dark-adapted retinas prepared in dim red light and from light-adapted eyes prepared in white light. The dark-adapted retina retained its deep pink

TABLE 1
THE EFFECT OF ALTERNATING LIGHT AND DARK
ON DARK-ADAPTED RETINAS

Expt.	No. Retinas	Temp. (° C.)	Dark			Light*			Dark		Light*
1.....	5	25.3	6	8	8	7	7	7	6	7	6
2.....	5	25.3	6	6	6	6	6	5	6	5	6
3.....	5	25.3	5	6	7	6	5	6	6.5	6	4.5
4.....	5	25.3	6	6	7	6	6	6.5	6.8	5.2	6
5.....	5	25.3	5	4	5	5.5	4	4.5	6	4	4

* 2,500-meter candle; readings made at 15-minute intervals and given in terms of millimeters change on manometer index scale.

color when placed in the manometer flask, whereas those prepared in the light became completely bleached during the process of preparation. Manometers containing from 5 to 25 retinas were tested for various periods of time in alternating darkness and light. The light for illumination was produced by several 100-200-watt electric bulbs. The intensities used in the different experiments ranged from 750- to 2,500-meter candle-power at the level of the manometer flasks. In most experiments readings were made every 10 minutes, and the results were computed in relation to comparable time intervals. In all, 67 experiments were performed, with results which proved essentially the same for both dark-adapted and light-adapted retinas. No measurable differences in the rates of oxygen consumption were found at different intensities within this range.

The results from this series are given in Tables 1 and 2. The data presented in Table 1 are typical of all experiments on dark-adapted retinas; and Table 2 gives the results of a

similar series on light-adapted retinas. In no case could a consistent difference in oxygen consumption between dark and light be demonstrated.

Several experiments were modified so as to lengthen the total exposure to dark and light. This was done in the case of darkness to allow more time for the regeneration of the visual purple. There was no indication that this procedure altered the rate of gaseous exchange.

In some cases, where as many as 25 retinas were used in each manometer and readings were taken at 5-minute intervals, there was some indication of an increased oxygen consumption during the period immediately following exposure to the dark. However, these results were not obtained consistently with the Warburg manometer and appeared to warrant further investigation. To check this point a modification of the Fenn manometer, having a scale reading of 0.001755 cc. per centimeter on the index capillary, was employed. A light intensity of 2,500-meter candle-power at the level of the flask was used. This intensity of illumination was sufficient to bleach the retina completely within

TABLE 2
THE EFFECT OF ALTERNATING LIGHT AND DARK ON
LIGHT-ADAPTED RETINAS

Expt.	No. Retinas	Temp. (° C.)	Light*			Dark				Light*			Dark
1.....	7	25.3	5.5	5.8	4.7	6.3	4	5	4.8	5.3	4.9	5.5	4.5
2.....	7	25.3	4	5	4.4	5.6	3.5	4.5	5.1	3.5	5	4	5
3.....	7	25.3	4	4	5.4	5.6	3.5	4.6	4.6	5.1	4	4.3	4.1
4.....	7	25.3	4.6	4.7	4.5	4.8	4.5	4.6	5	5.2	4.2	4.9	5
5.....	7	25.3	5.5	5.3	5.8	6.0	5.5	5.2	5.9	5	5.7	5.5	5.4

* 2,500-meter candle; readings made at 10-minute intervals and given in terms of millimeters change on manometer index scale.

10 minutes. From 1 to 4 retinas were used in each manometer, and readings were made every 5 minutes in alternating 20-minute periods of darkness and light. A very slight increase in the rate of oxygen consumption was usually obtained, lasting for about 10 minutes after the first exposure to darkness; however, subsequent changes from light to dark rarely gave any deviations from the value obtained for light.

With the foregoing data indicating that variations of light intensities, ranging from complete darkness to approximately 2,500-meter candle-power, exert no marked effect on the rate of respiration of the frog retina, a second series of experiments was designed to determine the amount of oxygen consumed by the retina. The oxygen consumption of from 2 to 10 retinas was determined during periods ranging from 2 to 4 hours at a light intensity of 750-meter candle-power. The solution in which the retinas were tested was the same as in the previous set of experiments. Approximately 400 retinas were used in 93 experiments. Table 3 presents the results of three groups of representative experiments taken at different times during the course of the investigation.

The data presented in the first two groups of experiments were obtained from paired retinas, whereas in the third group the retinas were removed and placed together before transferring them to the manometers. When retinas from different frogs are tested together, the rather large variation in metabolism so often found between different frogs is

not so apparent. The mean oxygen consumption for approximately 400 retinas tested in this series was found to be 3.22 ± 0.634 (S.E.) cu. mm. per milligram dry weight of tissue

TABLE 3
OXYGEN CONSUMPTION OF FROG RETINA AT $25^{\circ}3$ C.

Expt.	No. Retinas	Dry Wt. (Mg.)	Oxygen Consumed*	Expt.	No. Retinas	Dry Wt. (Mg.)	Oxygen Consumed*	Expt.	No. Retinas	Dry Wt. (Mg.)	Oxygen Consumed*
1.....	2	3.5	2.95	1.....	2	4.1	3.04	1.....	5	11.1	3.18
2.....	2	4	2.18	2.....	2	4.1	2.91	2.....	5	11.3	3.21
3.....	2	4	2.44	3.....	2	4.0	4.12	3.....	5	10.2	2.89
4.....	2	3	3.69	4.....	2	3.5	2.96	4.....	5	12.1	3.08
5.....	2	3.2	3.48	5.....	2	3.5	3.91	5.....	5	12.3	3.50
Total...	10			Total...	10			Total...	25		
Av.....			2.95	Av.....			3.39	Av.....			3.16

* Oxygen consumed in cubic millimeters per milligram dry weight of tissue per hour.

per hour. This compares quite favorably with the metabolism of brain tissue and ranks the retina as one of the most actively respiring tissues in the body.

SUMMARY AND CONCLUSIONS

1. The metabolism of the frog retina was studied with the Warburg method and a modified Fenn manometer.
2. The rate of oxygen consumption of the retina was determined for alternating periods in the dark and in the light.
3. The Warburg method reveals no consistent difference in the rate of oxygen consumption in the dark as compared with the light.
4. The data obtained with a Fenn manometer indicate a very slight increase in oxygen consumption for approximately 10 minutes after a light-adapted retina is exposed to darkness, followed by return to a value which remains constant during exposure to light.
5. The retina of the frog consumes an average of $3.22 \pm$ cu. mm. of oxygen per milligram dry weight of tissue per hour.

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THE EFFECT OF THYROID FEEDING ON OXYGEN CONSUMPTION OF THE GOLDFISH

(Two figures)

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THE influence of thyroid medication in raising the basal metabolic rate in mammals is well understood. The increase in metabolism is, indeed, so regular in some mammals—as, for example, man—that quantitative relationships could be standardized. Boothby and Sandiford (1928) state that each milligram of thyroxin injected increases metabolism by 2.8 per cent in myxedematous patients. From mammalian work the concept has arisen that a function of the thyroid hormone is to regulate the basic rate at which the processes of metabolism proceed. It is natural and easy for such a generalization to be extended from mammals to lower vertebrates, for structurally and chemically the thyroid gland appears to be much the same in all vertebrates. Yet, when this generalization is examined critically, it is seen to be based on little concrete evidence. The primary basis should rest on evidence that thyroid medication does increase the metabolic rate of the resting organism or conversely that thyroid removal lowers it. For the warm-blooded vertebrates this may be accepted as proved. The literature makes but scanty reference to any of the cold-blooded forms other than the amphibia. The reason for the extensive work in amphibia lies in the relation of this problem to the much-studied problem of the thyroid stimulation or metamorphosis. The usual interpretation (at least among American and English workers) of the work on the effect of thyroid on the metabolism of the metamorphosing amphibian has been that the metabolism is greatly accelerated but that this acceleration is not primary to the metamorphic activity (Helff, 1926). In 1934 one of us (Etkin) studied the oxygen consumption of tadpoles in normal metamorphosis. The results showed that during the growth phases of early metamorphosis the metabolism of the individual increased only in proportion to its growth. During the first days of the climax of metamorphosis, when the tadpole lost as much as 30 per cent of its former weight, the oxygen consumption of the animal likewise dropped and approximately to the same extent. Thus, per unit gross weight the metabolic activity seemed to remain constant. But it was further pointed out that the loss of weight in the animals in the climax of metamorphosis was almost entirely to be ascribed to loss of water from the tissues and to the loss of intestinal contents, the dry weight of the tadpole remaining about constant. When the entire desiccation curve was considered, it was evident that per unit dry weight the rate of metabolism of the tissues fell during all periods of metamorphosis and most rapidly during the metamorphic climax. Since the thyroid was believed to be active during metamorphosis and especially during climax (for general

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discussion see Etkin, 1936), it is evident that this conclusion is not consistent with the concept of metabolism control through thyroid activity. A critical re-examination of previously published evidence on thyroid and metabolism was then made, and it was found that the interpretation of other authors was based upon the fact that per unit gross weight the metabolism of animals artificially metamorphosed by thyroid show a distinct increase. However, the metabolism per individual dropped as in Etkin's data. The increase per unit weight reported by the previous workers was at least largely to be ascribed to the loss of water and intestinal contents rather than to actual increase in metabolism per unit dry weight. According to some data of Romeis (1920), there is a greater or speedier loss in water and intestinal contents in artificial than in normal metamorphosis. Whatever discrepancies appeared between Etkin's data on normal metamorphosis and the results reported by various experimenters on artificial metamorphosis seem to originate from this source. It seemed clear, therefore, that an interpretation of a thyroid effect on the metabolism of such animals was uncalled for and certainly equivocal.

Since 1934 there appears to be only one new study bearing directly on this problem, that of Wills (1936). In his study of oxygen consumption in various amphibia during development he reported increases in rate during metamorphosis. The chief concern of Wills's paper was with a study of metabolism in relation to sex differentiation. Though introducing several important advances in technique, particularly the use of protein nitrogen as basis for calculating the rate of metabolism, his treatment of the metamorphic problem suffers so much from a lack of appreciation of the literature and details of this process itself that it is difficult to evaluate his evidence. For example, though describing his techniques in considerable detail, he made no mention of the treatment of intestinal contents. Etkin previously pointed out the importance of this, at least for the frog, since at the beginning of the metamorphic climax in the tadpole the intestinal contents are evacuated. Thus an error which, in weight and presumably in nitrogen, may amount to more than 20 per cent is introduced, by not taking this loss of inert material into consideration. Even aside from the error of intestinal contents, his data on *Ambystoma* and *Rana pipiens* are too scanty to allow any clear inference or statistical evaluation. Indeed, the trend lines drawn through the points in his Figures 2 and 3 seem hardly consistent with the points themselves. Wills's few results on metamorphosis as given in Table 5 of his paper seem to us to be entirely consistent with the data and interpretation Etkin presented and indicate no fundamental increase in metabolism in the tadpole during metamorphosis.

It is evident that the problem in the metamorphosing amphibian must always be complicated by the profound morphological changes that take place under thyroid influence. The question of metabolism in cold-blooded animals would best be examined, therefore, where separated from metamorphosis. Henschel and Steuber (1931) have made an extensive study of thyroid injection and feeding in normal and in thyroidectomized frogs. Their findings were entirely negative; no effects of the experimental procedures on metabolic rate were found. This was apparently a thorough study, and its conclusions may be accepted as definitive for the adult frog. Drexler and Issekutz (1934) concluded that in a fish, a reptile, and two amphibia thyroid treatment does not raise the metabolic rate. Unfortunately, with regard to any particular form the evidence given is entirely too variable and scanty to justify much confidence in the conclusion. Perhaps, taken as a whole, the work justifies greater credence, but here again we are faced with personal opinion rather than with objective evidence.

The only other work (through 1939) known to the authors on the question of thyroid and metabolism of cold-blooded adult animals is found in the papers of Taylor (1937, 1939) on the salamander, *Triturus torosus*. He there developed the idea that the metabolism effect of thyroidectomy is at least partly secondary to the disorganization of the animal's nutrition. His data on oxygen consumption in thyroidectomized animals, calculated per gram total weight, show an initial slight increase for the first 16 days, followed by a more marked decrease 21-32 days after operation. It is not entirely clear whether, in view of the edema shown by the thyroidectomized animals, the oxygen consumption per animal or per unit dry weight would show any corresponding decrease or increase at any time. In a personal communication Taylor has suggested that there might not be any appreciable difference calculated per unit dry weight following thyroidectomy. Taylor thought to produce the hyperthyroid condition by transplantation of two pairs of thyroids. The results recorded indicate a profound drop in metabolism from the eighth to the twenty-fourth days and a great increase in the forty-sixth to the fifty-third days. The justification for considering that mere implanting of glands will induce hyperthyroidism may be questioned, especially since instead of the expected increase in molting these animals showed a decided decrease. In any case the irregularity shown by the results seems to the present authors to make it very difficult to give a physiological interpretation. This situation in the literature indicates the desirability of a new study of the relation of the thyroid to the metabolism of cold-blooded vertebrates. Such a study should (1) deal with an animal in which possible thyroid effects on metabolism are not accompanied by special morphological effects and (2) obtain data by a method permitting the establishment of standard conditions and make the data extensive enough to permit definitive statistical treatment.

Accordingly, we have set about to make such a study and have investigated the effects of thyroid on the metabolism of fishes. A preliminary report of our results with thyroxin administration in a single species of marine fish was reported earlier (Root and Etkin, 1937). It is our purpose here to report the results of a more extensive study on the common goldfish.

MATERIAL AND METHODS

A stock of about 150 goldfish was purchased from a commercial dealer. They were kept in large aquariums at 23°-25° C. for 3-4 weeks before experimental use. During this period they received the regular control food, which consisted of a mixture of dried shrimp, liver, lettuce, and oatmeal in about equal parts by weight.

The apparatus used was a continuous-flow type modified after Keys (1930a). A number of the modifications were copied from the apparatus used by Dr. Frank Schuett at the University of Chicago. In this apparatus a continuous flow of water passes through the animal container. Oxygen content of water samples was determined by the micro-Winkler method. From the difference between the oxygen content in the outflow from the animal chambers and that from a blank tube and the rate of flow, the oxygen consumption of the fish may be determined.

The structural details of our apparatus may be briefly summarized as follows: Cold New York City tap water was run through block-tin coils surrounded by running hot water. This served to warm the tap water, which then passed over a baffle system by which the oxygen content was brought to saturation at room temperature. The water was then led through a cotton filter to an overflow reservoir, which served to maintain a

constant head of water as a source of supply to the animal chambers, since it was kept overflowing at all times. From the reservoir the water was led through block-tin coils in the water bath to bring its temperature exactly to that of the bath. A distributor pipe then sent the water to each of 11 glass chambers. The animal chambers consisted of 8-inch lengths of $1\frac{1}{2}$ -inch glass tubing. The inlet was through the rubber stopper at one end, and the outlet in the corresponding place at the other end. The outlet was guarded by a fine-mesh wire filter. The outlets led through sections of capillary glass tubing to the bottom of the collecting bottles. The collecting bottles had a capacity of 35-45 cc. and a narrow neck only slightly larger in diameter than the capillary tubing. Such an arrangement meant that the overflow water from the collecting bottles must pass upward through a narrow space between the tubing and the neck of the bottle and would thus offset the possibility of any contamination of the sample with room air. We have checked this very simple collecting system with more elaborate ones and found it to be satisfactory. The collecting bottles were all arranged on a sloping shelf, and each sat in a metal tray that served to collect the overflow and concentrate it at one point, where it could be passed into a measuring flask for rate-of-flow determination. The rate of flow through the chambers could be regulated for the entire setup by a screw clamp at the outlet of the overflow reservoir or for individual chambers by varying the length of the capillary tubing. The water bath was provided with a cover that excluded almost all light from the animal chambers. Since in respiration measurements an experimental and a control group were always run simultaneously for comparison with each other, exact control of temperature was not deemed necessary. Temperature control was effected through the laboratory water-mixing valves. Only rarely did the temperature vary as much as $\frac{1}{2}^{\circ}\text{C}.$ during the course of a 3-day respiration period. Through the course of the work the temperature varied from 24° to $27^{\circ}\text{C}.$, but in the great majority of the readings (except low-temperature ones) it was about $26^{\circ}\text{C}.$

What must be emphasized here are the advantageous characteristics of this type of apparatus for the problem at hand. Perhaps most important of these is the possibility presented of allowing the animals to remain completely undisturbed in the chamber for days, while a series of readings is taken of their oxygen consumption. As will be seen later, this is a most important condition to be established if any standard metabolism measurements are to be attempted. Continuous-flow methods further avoid the difficulties and uncertainties inherent in the closed-system type because of the accumulation of the products of metabolism and the changing oxygen and carbon dioxide tensions. From the results of such studies as that on experimental confinement described later, it seems apparent to the authors that manometric methods involving shaking of the animal are out of the question because of the disturbance to the animals. In the present study the rate of flow for each fish was so adjusted as to result in the extraction by the fish of about half the oxygen of the water. This permitted the greatest accuracy, since the differential between animal and blank water was large, without, however, allowing the oxygen tension to drop to the point where it affects appreciably the rate of oxygen consumption (Keys, 1930b; Wells, 1935).

A point deserving of note is that the size of our collecting bottles, animal chambers, and the method of determining the rate of flow differ from those used by Keys and by Wells, both as regards the simplicity and ease of manipulation and as regards the kind of data yielded. From a calculation of the volumes of water involved it can be seen that our method gives a measure of the rate of oxygen consumption during the last few min-

utes before the sample was removed, whereas in Keys's method an average result over a period of several hours is given. The relative desirability of the two methods would of course depend upon the problem at hand.

The recent careful investigations of the Winkler method of determining dissolved oxygen (Allee and Oesting, 1934; Wilder, 1937) have shown the reliability of the method in dealing with uncontaminated waters. The suitability of the present water for this method has been checked by paired comparisons with the permanganate modification of the Winkler method, which is designed to remove interfering substances from the water. The results obtained by both methods were in good agreement.

The present report will discuss the results of five different types of treatment. These are (1) a preliminary determination of the effects of confinement, starvation, and external disturbances upon the oxygen consumption of the fish, (2) a paired comparison of the oxygen consumption of thyroid and control-fed fish, (3) a group comparison of such, (4) a comparison of the effect of a drop of temperature on the oxygen consumption of normal and thyroid-fed animals, and (5) effect of thyroid feeding on weight. The details of treatment in each series might better be discussed in connection with the results. In general, however, the following methods were pursued with regard to feeding and the taking of respiration readings. The control animals were fed two or three granules of fish food daily. The experimentals were fed on alternate days with fish food and with a single desiccated thyroid tablet (Burroughs Wellcomb & Co.)—each tablet representing 1 gm. fresh gland substance or 2/5 gr. U.S.F. powder. Each tablet was about equal in volume to the ration of fish food, so that the experimental animals received about half of their food supply as desiccated thyroid tablet.

Before being put into the respirometers, the animals were starved for 24 hours. Respiration readings were begun 18–24 hours after introduction. Two to 4 readings per fish were then taken daily for 2–4 days. The animals were left undisturbed and not fed during this period. After each such period of treatment the animals were returned to their aquariums for feeding periods of a week or more.

EXPERIMENTAL ANALYSIS

I. EFFECT OF EXPERIMENTAL CONFINEMENT AND LIGHT STIMULUS ON OXYGEN CONSUMPTION

A group of 20 animals was divided equally between two aquariums and fed control food for 1 week. Immediately after the last feeding the first 10 animals were introduced into the respiratory chambers, and O_2 -consumption measurements immediately begun. Eleven readings in all were taken over a period of 54 consecutive hours, 6 the first day, 2 the second day, and 3 the third day. On the morning of the third day, after the first respiration test, the black screen covering the apparatus was removed, and the animals exposed for half an hour to the light of a 100-watt bulb held just over the tank. Immediately after turning on the light, a reading was begun (i.e., the rate of flow determined), the sample bottles being removed between 15 and 20 minutes after the light was turned on. The cover was then replaced, and another reading taken 3 hours later.

In Figure 1 the results are given. It can be seen that from the first to the second hour the oxygen consumption increased slightly on the average but thereafter fell steadily during the rest of the first day (to 6 hours after introduction). At the twenty-fourth, twenty-eighth, and forty-eighth hours the rate was distinctly lower than on the first day

but seemed to have reached a fairly steady state about 35 per cent lower than the initial rate. This drop is clearly a real drop not to be ascribed to chance variation of the figures. The effect of the light stimulus was a 10 per cent increase in oxygen consumption followed by a decrease of about 20 per cent. The variability of the material is such that these smaller differences are not clearly significant. It can be seen that there is a tendency for the variability to be somewhat less on the second and third day than on the first.

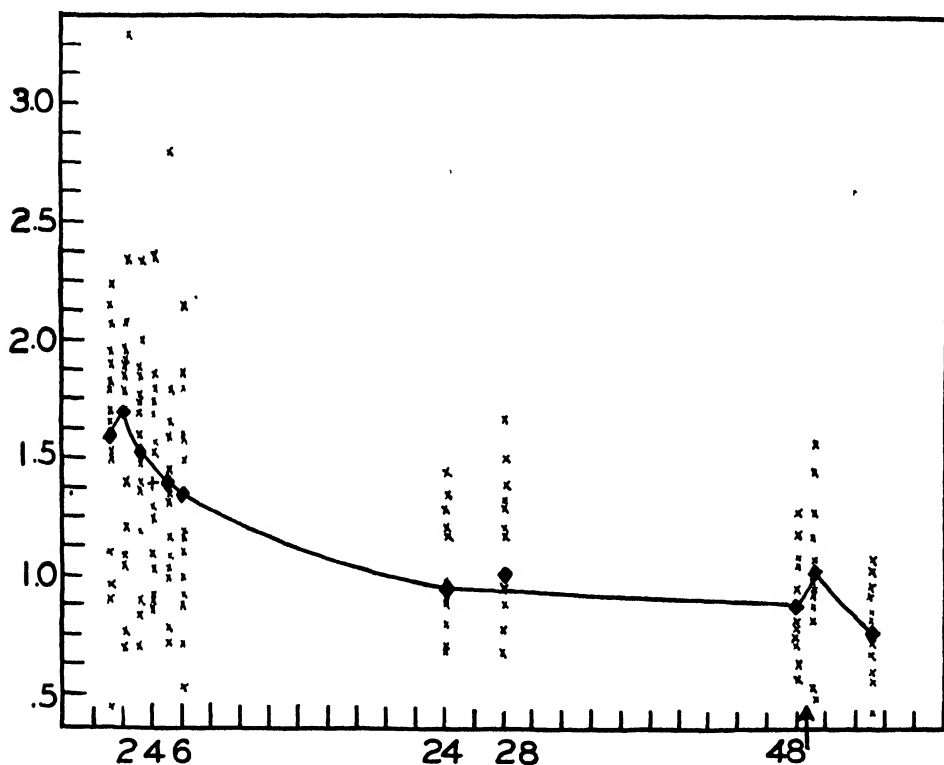


FIG. 1.—Rate of oxygen consumption in relation to time after introduction into respirometer; effect of light. Abscissae, hours after introduction; ordinates, cubic centimeters O_2 consumed per fish per hour. Arrow indicates time at which light was turned on.

The most noteworthy points brought out in this experiment are (1) the drop in metabolism in the first day and from the first to the second day, (2) the relatively steady metabolism during the second and third day, and (3) the small effect of the light as a disturbance. These results are very close to those of Wells (1932) in regard to the time factor.

It is, therefore, evident that for the standardization of metabolism reading it is very desirable that readings be taken only after the animals have been many hours in the apparatus. We have followed the practice of allowing 18–24 hours for the attainment of the steady state.

II. PAIRED COMPARISONS OF THYROID- AND CONTROL-FED FISH

After the preliminary weeks of adjustment to laboratory and food conditions the animals were selected in pairs, the members of each pair being as much alike as possible. The animals were placed individually in evaporating dishes, fed control food for a week, and then subjected to a preliminary period of oxygen-consumption measurements. In some cases two or three such periods were established before the experimental feeding was introduced. One member of each pair then was fed desiccated thyroid on alternate days, and the other continued on control food. Respiration tests were made as often as possible after the beginning of thyroid feeding, and the experiment continued as long as the animals seemed in good condition.

TABLE 1

RATIO OF OXYGEN CONSUMPTION IN PAIRED EXPERIMENTAL AND CONTROL FISH

No.	RATIO EXP./CONT. PRELIM.	RATIO IN EXPERIMENTAL PERIODS				
		3-10 Days	11-17 Days	18-24 Days	25-31 Days	32-38 Days
1.....	0.98±0.03	0.85±0.02
2.....	1.52±0.09	1.12±0.14
3.....	1.32±0.05	1.90±0.13	1.18±0.06
4.....	0.93±0.06	1.11±0.09
5.....	1.77±0.09	1.82±0.07	1.85±0.11
6.....	0.78±0.05	1.05±0.07
7.....	0.99±0.03	1.26±0.07	1.14±0.05
8.....	0.65±0.03	0.68±0.03	0.74±0.07
9.....	0.91±0.08	0.99±0.03	0.93±0.04	1.44±0.13	1.11±0.11	1.04±0.06
10.....	0.88±0.03	1.23±0.06
11.....	1.25±0.10	1.16±0.08	1.89±0.21
12.....	1.64±0.14	0.95±0.06	0.88±0.06	0.98±0.08	0.90±0.06	0.83±0.07
13.....	0.90±0.05	1.07±0.07

The results of the paired comparison experiments are summarized in Table 1. It can be seen that 13 cases are available for analysis, covering the period from 3 to 38 days after the beginning of experimental feeding, as well as the preliminary periods. Since the animals had been matched as to size, etc., the ratio of oxygen consumption in experimental and control animals was usually near 1. The figure given in the table is the average ratio for a series of 6-21 separate determinations made during the period in question. With each average is given its standard error. The experimental periods are classified into 5 groups according to the number of days after the beginning of experimental feeding that the readings were taken.

Even a cursory examination of the table shows that there was no constant alteration of metabolism associated with thyroid treatment. In some cases the ratio of the experimental to control decreased, in others it increased; but in the majority it showed no significant alteration. A statistical evaluation reveals that 10 of the 26 experimental periods show a significant deviation, 4 in a negative, and 6 in a positive, direction. (A probability of 0.01 was taken as the lower limit of significance.) Further evidence of the failure of thyroid materially to affect the metabolism of our fish is to be seen in the fact that where more than one experimental reading is available on one pair of fish there is no correlation

between the effect in one period and that in another. Thus the third pair shows a significant rise in the first week, followed by a fall in the second, which, however, is not statisti-

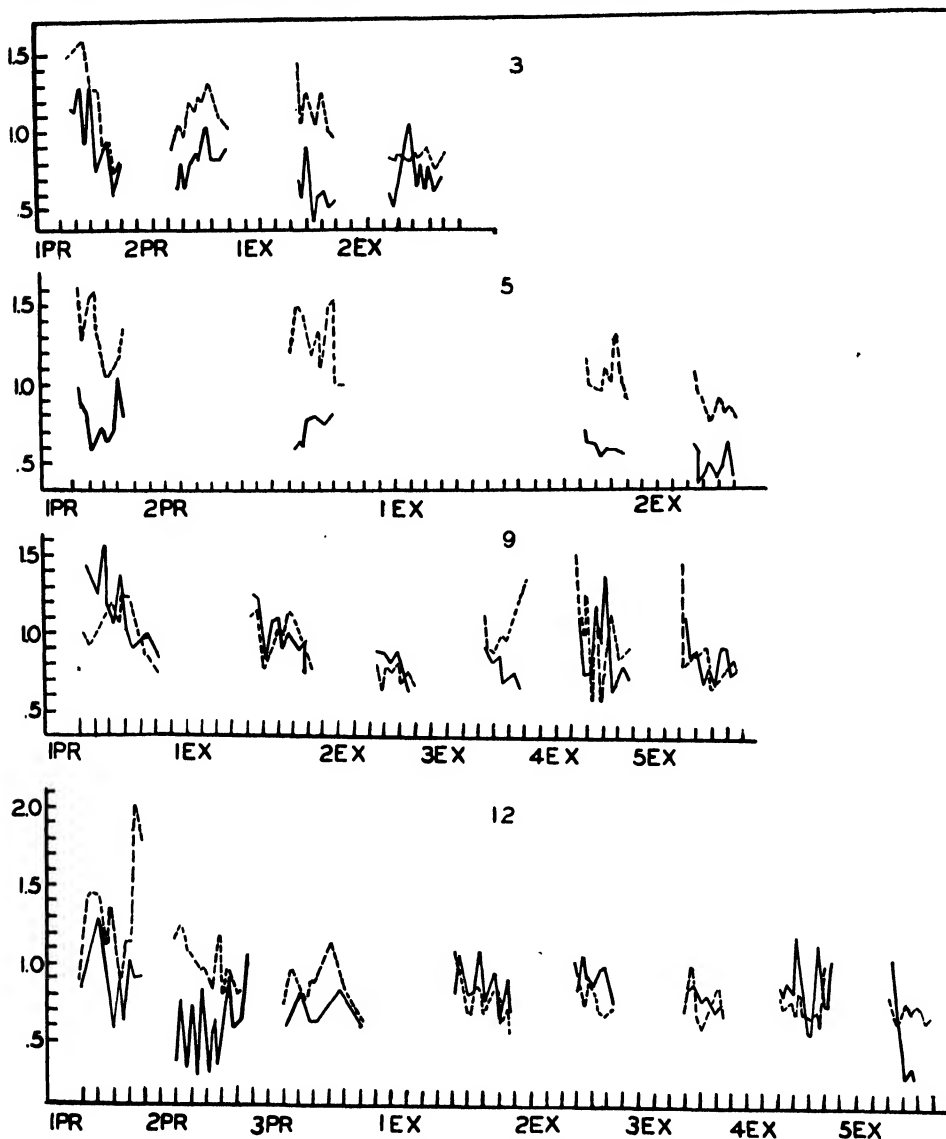


FIG. 2.—Rate of oxygen consumption in relation to thyroid feeding in four sets of paired comparisons. Ordinates, cubic centimeters O₂ per fish per hour; abscissae marked off in days. PR, preliminary period; EX, experimental period.

cally significant (see also Fig. 2). Only the seventh pair showing an increase and the twelfth showing a decrease exhibit any constancy of effect, and, of course, they do not

agree in direction. Finally, it should be noted that the evidence does not indicate any trend in the data with time, the periods showing significant variations being about evenly dispersed. Even after 5 weeks of thyroid feeding no specific effect appeared. Thus, the evidence indicates that the thyroid feeding had no demonstrable effect on the metabolism of the fish. In the seventh and twelfth cases, mentioned above as showing constancy in effect, the effect did not increase with time.

One point in regard to statistics is deserving of some comment. It might be objected that 10 deviations out of 26 tries is rather high on the calculated basis that each had a probability of 0.01. But it must be recalled that the variance upon which the calculation of probability is based is taken from the data within each experimental period. The variability from one period to the next is not taken into account. The large number of significant deviations may be taken to indicate that a real change often takes place in the relative rate of metabolism of the experimental and control animals, our previous remarks being to the effect that this real change is not ascribable to the influence of thyroid.

TABLE 2
OXYGEN CONSUMPTION IN RELATION TO TIME AFTER BEGINNING OF THYROID FEEDING*

TIME	A		RATIO	B		RATIO
	Control	Exper.	Exper./Control	Control	Exper.	Exper./Control
Preliminary...	0.99±0.05	1.07±0.06	1.08	1.15±0.08	1.13±0.07	0.98
4 weeks.....	0.80±0.04	0.84±0.05	1.05	1.11±0.06	1.15±0.07	1.04
6 weeks.....	0.91±0.08	0.93±0.05	1.02
7 weeks.....	0.66±0.04	0.73±0.05	1.10
9 weeks.....	0.71±0.04	0.70±0.05	0.99

* Oxygen consumption given as cubic centimeters O₂ per fish per hour.

This is, perhaps, best appreciated in an examination of a graphic representation of the data for some of the pairs of animals as in Figure 2. In the first group (pair No. 3) the solid line representing the control animal showed a distinct drop after the first experimental period but came up again after the second period of experimental feeding. The experimental, on the other hand, remained about the same or rose slightly after the first period and fell at the second. It is thus apparent that in this particular case the significant change seen in Table 1 after the first thyroid feeding is to be ascribed more to the fall in the control rather than to a direct rise in the thyroid-fed animal. The results plotted in the other cases reveal again cases where a relative change occurred in the metabolism of experimental and control which, though clearly real enough, cannot be ascribed to any metabolism effect of thyroid. In the second pair plotted (pair No. 5) it is apparent that both animals clearly tended to fall off in metabolic rate with the passage of time, in this case at much the same rate. A general tendency in this regard was observed in all our animals, as, for example, in the graphs here given and in the data of the group experiments in Table 2.

Thus far our interpretation of results leads us to the conclusion that by the technique applied in this study no effect of thyroid feeding on oxygen consumption could be demonstrated. Such a conclusion is, of course, all that could properly be expected in the interpretation of negative evidence. Yet it is interesting to ask whether we have any internal

evidence as to how accurate our study is, how large a deviation it would detect, or, stated conversely, how large a difference which, if present, would have been clearly revealed by our methods. It is possible to make a calculation of the amount of deviation which, with data of the variability of our material, would give a significant difference from the mean of the preliminary period. Such a difference may be expressed as percentage of the mean.²

The average figure found for this percentage was 34 per cent. In 18 of the 26 cases it did not exceed the average, being as low as 12 per cent in 1 case. It is thus apparent that under the conditions of these experiments any factor which raised or lowered the metabolism of a particular fish by as much as 34 per cent would be definitely detectable in a single period of average variability. A trend in a group of cases such as here presented would, of course, be apparent even if the influence of the factor responsible were considerably less than 34 per cent.

As we have seen above, no influence or even definite trend was found in the thyroid-fed animals. We may conclude, therefore, not only that our results showed no influence of thyroid feeding on metabolism, but, more, they showed that any influence that may have escaped detection in the present experiments could have been of only minor significance with an effect of considerably less than 34 per cent.

III. GROUP COMPARISON OF THYROID- AND CONTROL-FED FISH

It was felt that the paired comparison experiments described above suffered most from the fact that the animals did not usually survive well for more than a few weeks. The frequent confinements in the respirometers under starvation conditions were judged the chief cause of this, though the animals also did not seem to remain completely healthy, merely as a result of the necessary isolation in evaporating dishes. A group experiment was therefore designed to overcome these deficiencies.

In this experiment 40 animals from the stock aquariums were selected and grouped into 4 groups of 10 animals each. The selection was made so that 2 sets of experimental and control groups could be made, the members of each set being of about the same size. All animals were fed control food for 2 days and then placed in the respirometers for a set of preliminary readings. They were then returned to their aquariums, and the differential feeding of control and experiment groups was begun. The experiment was continued for 7 or 9 weeks, with oxygen-consumption readings being taken at the fourth, seventh, and ninth week. The results are given in Table 2.

It should be remarked that the average given for each period was found by using as individual values the mean of the 5 or 6 determinations for each individual fish. These figures were then averaged, and the standard error calculated as recorded in Table 2.

² The formula used in calculating the statistical significance of the difference of the means of the preliminary and experimental periods is taken from Fisher (1925). It is

$$t = \frac{(x_1 - x_2) \sqrt{N_1 + N_2 - 2}}{\sqrt{S_1 + S_2}} \sqrt{\frac{N_1 N_2}{N_1 + N_2}}.$$

The probability *P* was derived from "Students" distribution for the calculated *t* and the appropriate degrees of freedom. For the calculation of the size of deviation that our methods would reveal, the *t* corresponding to the assumption of a probability of 0.01 was obtained from the table, and the equation solved for the value of (*x*₁ - *x*₂). This value was then expressed as percentage of *x*₁. The data on variability used in this calculation was that supplied by the readings for the preliminary period and for the experimental period in question.

The significance of this method is that the measure of variability given represents the variability between fish and is not mixed with measures of the variability of successive readings of the same fish. It can be seen that in each series there is a tendency for the O_2

TABLE 3
THREE EXPERIMENTS ON THE EFFECT OF A DROP IN TEMPERATURE
IN THYROID-FED AND CONTROL GOLDFISH*

Day	Temperature (° C.)	Thyroid-Fed	Ratio High/Low	Control-Fed	Ratio High/Low
Experiment A					
I.....	25	0.69 ± 0.13	0.65 ± 0.11
I.....	25	0.64 ± 0.09	0.62 ± 0.11
I.....	14.5	0.21 ± 0.05	0.32	0.19 ± 0.05	0.30
2.....	14.2	0.25 ± 0.03	0.38	0.21 ± 0.03	0.34
2.....	15.5	0.23 ± 0.02	0.35	0.21 ± 0.03	0.34
3.....	15	0.26 ± 0.04	0.39	0.25 ± 0.04	0.38
3.....	15.2	0.26 ± 0.04	0.39	0.26 ± 0.04	0.41
Experiment B					
I.....	25	1.12 ± 0.10	0.76 ± 0.05
I.....	25	1.21 ± 0.09	0.84 ± 0.16
2.....	24	1.06 ± 0.09	0.74 ± 0.18
2.....	13.5	0.41 ± 0.06	0.36	0.27 ± 0.05	0.35
2.....	13	0.24 ± 0.04	0.21	0.26 ± 0.03	0.33
3.....	14.5	0.38 ± 0.02	0.37	0.28 ± 0.05	0.36
3.....	15	0.28 ± 0.07	0.25	0.27 ± 0.03	0.35
4.....	14.5	0.34 ± 0.03	0.30	0.28 ± 0.02	0.36
Experiment C					
I.....	24	0.73 ± 0.07	0.67 ± 0.03
I.....	26.9	1.03 ± 0.06	0.82 ± 0.06
2.....	25	0.87 ± 0.07	0.83 ± 0.07
2.....	13	0.29 ± 0.02	0.33	0.26 ± 0.03	0.34
2.....	15	0.18 ± 0.06	0.21	0.22 ± 0.06	0.29
3.....	15	0.41 ± 0.09	0.47	0.27 ± 0.03	0.35
3.....	15	0.34 ± 0.03	0.39	0.30 ± 0.04	0.39
4.....	15	0.33 ± 0.03	0.38	0.27 ± 0.03	0.35

* Oxygen consumption given as cubic centimeters O_2 per fish per hour.

consumption to drop off considerably with the passage of time. This in spite of the fact that the animals remained very active and healthy in appearance throughout. It will be noticed, however, that no significant difference in the amount of drop appeared as between the thyroid- and nonthyroid-fed animals. The ratio between these dropped some-

what in the first series, at least at the ninth week, and rose slightly in the second series. Both changes, however, are clearly within the limits of error of the experiment. It is, therefore, apparent that in these experiments running for as long as 7-9 weeks of thyroid feeding no significant effect of the experimental feeding could be detected.

IV. TEMPERATURE EFFECT

Huxley (1929) has speculated on the possibility of the thyroid's acting as a temperature buffer to reduce the accelerating action of heat on the rate of metabolism of cold-blooded animals. According to this notion, the activity of the gland (or perhaps the reactivity of the tissues to the hormone) decreases as the temperature increases; thus the loss of thyroid stimulation when the temperature is raised decreases the total effect of the temperature increase. The possibility becomes apparent that the failure we experienced in detecting any effect of thyroid on O_2 consumption could possibly be ascribed to the rather high temperature ($26^\circ C.$) at which the experiments were run. We, therefore, determined to study the effect of a drop of temperature on the O_2 consumption of control- and thyroid-fed animals.

A group of experimental animals were paired with controls and fed according to the experiments previously described. In the first experiment (Table 3, A) the animals surviving from the group experiment were used. In these the experimentals had been fed thyroid for over 7 weeks; in the other experiment the animals were differentially fed for 3 and 2 weeks, respectively. The animals were introduced into the respirometers in the afternoon, allowed to rest undisturbed over night, and the next day two readings of O_2 consumption were taken. In the first experiment the temperature was quickly lowered in the entire apparatus, and another reading was taken between 2 and 3 hours thereafter. In the later experiments the drop in temperature was delayed another day, giving time for an early morning determination at high temperature and two determinations at lower temperature. Readings were continued to the third day after dropping the temperature. An examination of Table 3 shows that the drop of approximately $10^\circ C.$ in temperature resulted in a drop to approximately $\frac{1}{2}$ in O_2 consumption. This drop was pretty well maintained for the 3 days of the experimental period, though in the first experiment a slight tendency of the values to rise might be inferred. It is, however, abundantly clear that the thyroid-fed animals behaved in all respects very much like their controls, no constant difference appearing at any time. In short, the temperature coefficient of O_2 consumption in these animals did not seem to be affected by the period of thyroid feeding that had gone before.

V. EFFECT OF THYROID FEEDING ON WEIGHT

It would, of course, have been desirable to follow the weight changes on the same animals as were used in the respiration studies. This, however, proved impracticable, since it was found difficult to keep the fish in good condition and the added handling involved in weighing clearly was detrimental to them. Weighing the fish used in respiration studies was therefore soon abandoned, and recourse was had to a separate experiment on this point. An added point to be established by a study of this kind was the question of the nutritive adequacy of the control and thyroid feeding. Ten groups of 5 fish each were set into aquariums. These groups were made up in pairs, each member of the pair having about the same average and range of weight. The animals were fed for 1 week on control food and then weighed. One group of each pair was then subjected to the thyroid-feed-

ing schedule, and the other group served as control. Weighings were again made at the fourth, ninth, and sixteenth weeks after the preliminary period. A standardized handling was developed to cut down as much as possible on the variability. This consisted of shaking the excess water off the fish by three swings of the arm and then weighing in water in a tarred cardboard container.

The results of this experiment are shown in Table 4. Inspection of the figures shows that, in general, the control fish maintained their weight pretty well but did not gain or lose much. The experimentals showed much the same behavior. Occasionally as in group 10 there was some loss, or as in group 4 consistent gain, but, on the whole, the picture closely duplicates that of the controls. It is thus clear that a diet consisting of approxi-

TABLE 4
EFFECT OF THYROID FEEDING ON WEIGHT IN GOLDFISH*

DURATION OF THYROID TREATMENT	SET 1		SET 2		SET 3		SET 4		SET 5	
	Control	Exper.	Control	Exper.	Control	Exper.	Control	Exper.	Control	Exper.
Prelim.	12.61	12.56	15.03	14.85	11.15	11.14	11.15	11.21	14.33	14.20
4 weeks.	12.55	12.34	14.85	16.03	11.69	11.75	11.30	11.30	13.80	14.34
9 weeks.	12.49	12.29	14.06	15.51	11.77	11.64	11.22	11.19	13.51	12.85
16 weeks.	12.48	12.48	13.65	15.22	11.82	11.52	11.28	11.16	13.84	14.38

* Figures give average weights in grams of the 5 animals in each group.

mately one-half of a dried thyroid tablet does not lead to any marked alteration in growth or weight maintenance as compared with our control food. It is clear that no marked difference in the nutritional value of the two types of diet appeared. No adjustment of the respiratory trends for weight changes or for other nutritional factors is called for.

DISCUSSION

The negative results of thyroid feeding upon the oxygen consumption and the weight of goldfish here reported are not consistent with the commonly held opinion that thyroid substance is a general stimulant to cell metabolism. They are, however, in agreement with the results of the two European investigators mentioned in the introduction and are further consistent with the interpretation one of us had previously made of the more extensive literature on amphibian metamorphosis in relation to thyroid and metabolic rate. We feel justified, therefore, in concluding that the effect of thyroid on general metabolism of mammals and birds may not properly be extended to any of the cold-blooded animals that have been worked on, including fish and adult amphibia.

It may always be said that such negative results as are reported in this paper may be due to a technical failure of one kind or another. We believe that we have disposed of the objection that our technique was not capable of detecting a small effect by showing in a number of ways that the apparatus was capable of yielding clear-cut results on effects amounting to from 10 to 30 per cent. If a diet containing as much thyroid as our experimental feeding does not produce a detectable effect under these conditions, it seems hardly possible to think of the thyroid as of any considerable physiological importance in the regulation of the rate of oxygen consumption in these animals. Mere illumination of the

animals without further disturbance produces a detectable physiological effect which thyroid feeding does not do.

Perhaps a more significant objection to an interpretation that denies a role in metabolism regulation to the thyroid in cold-blooded animals lies in the fact that we (and all other workers) fed mammalian thyroid to nonmammalian forms. It would indeed be very interesting to compare nonmammalian forms in their response to their own and to mammalian thyroid. Until this can be done, however, we must be content with pointing out that Henschel and Steuber (1931) found no metabolic effect of thyroidectomy in frog; that mammalian thyroid is effective in other regards on cold-blooded animals, i.e., amphibian metamorphosis, amphibian and reptilian skin shedding; and that Artundo and Solari (1933) found that anterior pituitary extracts effective on the dog were ineffective in raising the basal metabolic rate of toads (despite the fact that it induced thyroid stimulation in the toad). What evidence exists negates the idea that the thyroid plays any considerable role in regulating the rate of metabolism in cold-blooded animals.

The role of the thyroid in homoiothermal regulation has long been the subject of considerable investigation. The absence of a metabolic effect of the thyroid in cold-blooded animals might be taken as indicating the special significance of the thyroid for temperature regulation. A general discussion of this problem seems out of the province of the present paper, but the somewhat analogous notion, ingeniously developed by Huxley (1929), that the thyroid serves as a physiological buffering device against changes in temperature in cold-blooded vertebrates deserves further consideration. It was felt that, if this were indeed the case, a difference in the ability of the fish to adjust itself to a sudden lowering of the temperature might be expected as between control and thyroid-fed animals. As noted above, we were unable to detect any difference between the control and experimental fish in this regard. We do not regard this test as a sufficient exploration of the possibilities of the idea. Yet the results do not encourage any confidence in its further development.

In view of the failure of the evidence from many sources and of many varieties to show any metabolic rate regulating effect of the thyroid on cold-blooded animals we are inclined to the view that such a relationship is peculiar to the physiology of the homoiotherms. The physiological importance of the thyroid in the economy of the cold-blooded vertebrates lies perhaps in the specific tissue responses as metamorphosis, skin-shedding, etc., which are known in some animals.

SUMMARY

A study of the effect of thyroid feeding on the oxygen consumption of goldfish was made. Desiccated mammalian thyroid was fed to the extent of about half the food supply of the experimental animals. The methods of metabolism determination permitted the attainment of a high degree of standardization and of adequate statistical evaluation. The following results were found.

1. During the first 6 hours after introduction into respirometers the oxygen consumption of normal undisturbed goldfish fell steadily but during the second and third days maintained a steady state about 60 per cent of the initial value. Exposure to light seemed to raise the metabolism about 10 per cent.

2. Animals fed thyroid tablets to the extent of half their food supply did not reveal any increased metabolism even after 9 weeks. A statistical evaluation of the results indicates that an effect of considerably less than 30 per cent would have been clearly revealed.

3. The effect of a drop of temperature on oxygen consumption was the same in control- and in thyroid-fed animals.

4. Thyroid feeding had no effect on the weight of fish even when continued for 16 weeks.

The results are interpreted as supporting the notion that the metabolism-accelerating effect of thyroid substances as found in mammals is absent in goldfish.

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NUTRITIONAL STUDIES OF PARAMECIUM MULTIMICRONUCLEATA

II. BACTERIAL FOODS

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PURELY experimental studies on the growth and reproduction of *Paramecium* have been limited to the last fifty years. Observations of isolated cultures of these organisms, however, were made as early as 1830 by Ehrenberg, who followed the multiplication of 2 single individuals in clear water through a 10-day period. During the first 6 days "these polygastric Infusoria existed without their reproductive power being developed," but by the end of the tenth day these animals had multiplied to such an extent that it was impossible to count them. In 1860 Balbiani estimated that a single *Paramecium* would produce 1,384,416 offspring in the course of 42 days. He obtained this figure by multiplying together the progeny of several successive subcultures which had been isolated in hay infusions.

In 1888 Maupas criticized the observations of these early investigators in that the importance of the food factor in their studies on growth and reproduction was overlooked. Discussing the nutrition of protozoa, he said: "Perhaps by employing the methods of Pasteur for the pure culture of Schizomycetes, one would succeed in finding one still more appropriate to the needs of the Ciliates." However, he did not make any attempt to find such a food organism, nor have many others in their experimental studies of protozoa.

Hargitt and Fray (1917) were the first to use bacteriological methods in making a detailed study of the food requirements of *Paramecium*. The bacteria chosen as food organisms were those which appeared to be dominant at different periods of an aging hay infusion. Of the 30 bacteria isolated in pure culture, 11 were partially identified. From their findings Hargitt and Fray concluded that mixed cultures of bacteria are, as a rule, far superior to a single strain as a diet for *Paramecium*. The conclusions of Phillips (1922) on feeding *Paramecium* known bacteria substantiated those of Hargitt and Fray.

Cleveland (1928a) criticized the work of Hargitt and Fray and that of Phillips by stating:

They have attempted to grow the paramecia in too few pure cultures of bacteria for the investigation to have much value. Had they used more pure cultures of bacteria, it is very probable that quite different results would have been obtained. Instead of finding, as they did, that *Paramecium* grew better in mixed cultures of certain bacteria than in pure cultures, it is most likely that they would have found it grew better in pure cultures of certain bacteria—perhaps far more abundantly than in mixed cultures.

He also pointed out that their work was limited by the lack of most of the usual bacteriological facilities.

Owing either to the suggestion of Cleveland or to the realization that bacterial feeders can thrive on a single strain of bacteria, recent investigators studying paramecia experimentally have usually employed only pure cultures of bacteria as food for this ciliate. However, a survey of the literature to determine a satisfactory food for paramecia

TABLE 1
SUITABILITY OF BACTERIA AS FOOD FOR SPECIES OF *Paramecium* AS
REPORTED BY VARIOUS INVESTIGATORS

Investigator	Paramecium	Culture Medium	Food Organism	Suitability as Food
Hargitt and Fray, 1917....	caudatum aurelia	Organic Organic	Micrococcus flavus	—
			Bacterium plicatum	—
			Bacillus Raveneli	—
			B. dendriticus	—
			B. guttatus	—
			B. fluorescens	—
			B. flavescens	—
Phillips, 1922.....	aurelia	Organic	B. subtilis	±
			B. coli	—
			B. cereus	—
Philpott, 1928.....	aurelia calkinsi caudatum	Organic	B. proteus	—
			B. pyocyaneus	+
			B. enteritidis	—
Pringsheim, 1928.....	caudatum	Organic	B. proteus	+
			Azotobacter	—
Chejfec, 1929.....	caudatum	B. coli	+
Raffel, 1930.....	aurelia	Organic*	B. candicans	+
Damerow, 1931.....	caudatum	Organic	Tubercle bacilli	—
Losina-Losinsky, 1929 and 1931.....	caudatum	Organic	B. fluorescens liquefaciens	—
			B. coli communae	—
			B. subtilis	+
Hetherington, 1934.....	caudatum	Achromobacter pinnatum	+
Phelps, 1934.....	aurelia	Organic*	Erythrobacillus prodigiosus	+
Giese and Taylor, 1935...	multimicronucleata	Organic*	Pseudomonas ovalis	+
Johnson, 1936.....	caudatum	Inorganic	Escherichia coli	—
			Aerobacter aerogenes	—
			B. subtilis	+
			Ps. fluorescens	—
			Serratia marcescens	—
			Staphylococcus aureus	—
Giese, 1938.....	multimicronucleata	Organic*	Ps. fluorescens	±
			B. subtilis	±
Johnson and Hardin, 1938..	multimicronucleata	Inorganic	Ps. fluorescens	+
De Lameter, 1939.....	aurelia	Organic*	Flavobacterium brunneum	+
			B. niger	+
			B. cereus	+
			B. prodigiosus	—
			B. coli	—

* Considered organic when either lettuce or algae is used in connection with the bacteria.

shows that there may be but little agreement as to the suitability of a given food organism. Table 1 summarizes the findings of investigators as to the food suitabilities of various bacteria introduced as pure cultures into an organic or inorganic culture medium. From this table it is evident that different investigators do not always report similar findings as to the food qualities of a specific bacterium. Such a lack of understanding of the basic problem of protozoan nutrition in a form so much investigated as *Paramecium* is unfortunate.

Studies of the foods of such bacterial feeders as *Tritrichomonas fecalis* and *Glaucoma ficaria* have been made by Cleveland (1928b) and Johnson (1936), respectively, and have aided materially in our understanding of the nutritional requirements of these protozoa, as a large number of bacteria were tested as food organisms in each case. Luck, Sheets, and Thomas (1931) have reported nutritional studies on *Euplotes taylori*, feeding on a very limited number of isolated pure strains of bacteria, as has Hetherington (1933) for *Colpidium campylum* and *C. colpoda*. The importance of the food factor was clearly stressed by Jahn (1934), when he pointed out the numerous factors affecting the growth of protozoa. And Hammond (1938), who has made a review of the literature dealing with the role of foods in relation to allelocatalytic phenomena, indicates the significance of understanding the food factor.

Recently it has been demonstrated that *Pseudomonas fluorescens*, a bacterial food of *Paramecium multimicronucleata*, can be qualitatively and quantitatively standardized, facilitating reproducible results in experimental studies on this ciliate (Leslie, 1940). Using these same techniques, it was decided to try to determine the relative suitability of some 30 species of bacteria as food organisms for *P. multimicronucleata*.

MATERIALS AND METHODS

The standardization of experimental cultures of *Paramecium* involves the use of (1) Osterhout's non-nutrient culture medium, (2) a pedigreed strain of food bacteria qualitatively controlled as to age and quantitatively measured by the wire-loop method, and (3) controlled optima for all physical factors normally considered.

The food organisms selected for the present investigation were pedigreed strains obtained from the bacteriology department of Stanford University. All these foods were qualitatively standardized by cultivating the bacteria for 24 hours at optimum temperatures on slants of the commercial Difco Bacto nutrient agar except for the species of *Mycobacteriaceae*, which were cultured for 48 hours because of their slower growth. The reason for cultivating the bacteria either 24 or 48 hours before use was to insure a large number of living bacteria in each food suspension. The necessity of daily subculture of the bacteria required that the purity of all the cultures be regularly checked throughout the course of the experiment; none of these became contaminated. At the time of feeding the paramecia, two level loopfuls of the qualitatively standardized (24- or 48-hour-old) bacteria were removed from the slant with a 1-mm. platinum loop and suspended in 5 cc. of the sterile salt solution serving as the culture medium.

The ratio of the volume of the culture medium to the number of paramecia at the time of subculture was constant at 1 cc. to 20 animals. Sterile volumetric pipettes were used to carry 1 cc. of the bacterized culture medium into clean ground Columbia culture dishes of 1-cc. capacity, in which the ciliates were grown as isolation cultures.

The paramecia were subcultured daily by isolating 20 with a sterile-mouth pipette from a similar culture started 24 hours earlier and grown in a glass moist chamber main-

tained at 26°–27° C. Daily counts of the animals in each 1-day-old subculture were made under a dissecting microscope, with counting facilitated by the use of a mechanical hand counter. From the total number of progeny produced over a 4-week period the average daily fission-rate of the ciliates was determined in each of the foods tested. These rates served as an index of the suitability of the various food organisms.

No dust-protecting hood was employed during the daily subculture of the ciliates into the bacterized inorganic culture medium, as is usually the case when an organic culture medium is used. As will be pointed out later, no bacterial contamination resulted from this procedure. This was to be expected, as W. H. Johnson (1933) previously reported that this medium will not support the growth of the bacterial inoculum.

The control food for these experiments was *Ps. fluorescens*, which was cultivated for 4 days on the nutrient agar before being suspended in the salt medium. Unfed paramecia in a 1-cc. volume of sterile salt solution were also subcultured daily, and these permitted a rough control in comparing the suitability of the various food organisms. The animals used in this study were taken from a mass culture of *P. multimicronucleata* which Professor W. H. Johnson has had growing in his laboratory at Stanford University for many months at 25° C. in Osterhout's inorganic culture medium on *Ps. fluorescens*.

EXPERIMENTAL DATA

The species of bacteria chosen as possible food organisms for *P. multimicronucleata* are nonpathogenic representatives of several different families. Although the number of selected bacteria was as large as possible to work with satisfactorily, there were still too few species tested to make comparisons between the families. The present investigation was designed merely to determine the various bacteria as food organisms under standardized experimental conditions.

In order to determine the suitability of these bacteria the degree of acclimation of the ciliate to them was used as a criterion, the degree of acclimation being the average daily fission-rate which the paramecia maintained in the standardized bacterial suspensions over a 4-week period. If any of the subcultures failed to become acclimated to a particular bacterium and consequently died out during the course of the experiment, such cultures were replenished by washed animals from the mass culture. Thus all the parental cultures were assumed to be comparable.

Owing to the number of parallel cultures maintained, it was possible to carry only a single-animal culture in each of the different food suspensions. Hence the suitability of the various bacteria as food organisms has been estimated according to the average daily fission-rate of the paramecia in single-line cultures as follows:

1. Division-rate 0.0–0.9 food organism POOR
2. Division-rate 1.0–2.0 food organism GOOD

The division-rate was calculated from the progeny of the 20 paramecia subcultured daily. If after 24 hours of cultivation the progeny numbered 40, the division-rate of the animals during that period would be 1.0, since each of the organisms had apparently divided once. Had the number of offspring been 60 or 80 during the same period, the division-rate would have been 1.5 or 2.0, respectively. From the average daily number of individuals present in each subculture the average daily fission-rates were calculated and the suitability of the food organisms estimated. Table 2 summarizes these findings according to (a) classification number of the bacteria, which is that of the bacteriology de-

partment of Stanford University, (b) scientific name of the bacterium, (c) total number of progeny, (d) duration of the experiment in days, (e) average number of offspring produced per day from each subculture, (f) average daily fission-rate of the paramecia, and (g) rating of the suitability of the bacteria as food organisms.

TABLE 2
SUITABILITY OF BACTERIA AS FOOD FOR *Paramecium multimicronucleata*
CULTURED IN AN INORGANIC MEDIUM

Cat. No.	Bacterium	Total Progeny	Days	Av. No.	Division Rate	Suitability
D-2.....	<i>Bacillus mycoides</i>	832	27	30	0.5	Poor
D-10.....	<i>B. cereus</i>	1,303	29	44	1.1	Good
D-20.....	<i>B. megatherium</i>	1,967	29	67	1.6	Good
D-21.....	<i>B. mesentericus</i>	894	27	33	0.6	Poor
D-28.....	<i>B. niger</i>	1,068	29	36	0.8	Poor
D-41.....	<i>B. terminalis</i>	942	27	34	0.7	Poor
D-39.....	<i>B. subtilis</i>	655	15	43	1.0	Good
D-40.....	<i>B. subtilis</i>	322	14	23	0.1	Poor
K-11.....	<i>Escherichia coli</i>	1,034	29	35	0.7	Poor
K-12.....	<i>E. coli</i>	774	30	25	0.2	Poor
A-5.....	<i>Aerobacter cloacae</i>	1,711	29	59	1.4	Good
A-6.....	<i>A. aerogenes</i>	1,372	29	47	1.1	Good
Al-2.....	<i>Erwinia carotovora</i>	1,508	29	51	1.2	Good
Al-1.....	<i>E. atroseptica</i>	789	30	26	0.3	Poor
C-1.....	<i>Alcaligenes faecalis</i>	1,670	29	57	1.4	Good
T-6.....	<i>Proteus vulgaris</i>	1,224	27	45	1.1	Good
Z-1.....	<i>Serratia marcescens</i>	1,260	27	46	1.1	Good
AA-2.....	<i>Spirilla serpens</i>	1,077	30	35	0.7	Poor
Ad-13.....	<i>Staphylococcus albus</i>	597	30	19	0.0	Poor
AM-1.....	<i>Phytomonas savastanoi</i>	569	30	18	0.0	Poor
AM-3.....	<i>P. tumefaciens</i>	1,910	30	63	1.6	Good
AO-1.....	<i>Cellulomonas biazotea</i>	618	30	20	0.0	Poor
F-1.....	<i>Actinobacillus lignieresii</i>	645	30	21	0.0	Poor
G-17.....	<i>Corynebacterium ovis</i>	702	30	23	0.1	Poor
N-1.....	<i>Micrococcus luteus</i>	547	30	18	0.0	Poor
N-2.....	<i>M. ureae</i>	731	30	24	0.2	Poor
O-6.....	<i>Mycobacterium phlei</i>	779	30	25	0.2	Poor
O-15.....	<i>M. smegmatis</i>	803	30	26	0.3	Poor
O-21.....	<i>M. berolinense</i>	752	30	25	0.2	Poor
U.....	<i>Pseudomonas ovalis</i>	2,117	30	70	1.7	Good
U-6 (5 lines)*..	<i>Ps. fluorescens</i>	1,043	43	24	0.2	Poor
U-6 (5 lines)†..	<i>Ps. fluorescens</i>	2,462	41	60	1.5	Good

* 24-hour-old bacteria.

† 4-day-old bacteria.

DISCUSSION

The present findings indicate that among the food organisms selected only about one-third proved suitable for *P. multimicronucleata* cultured under these conditions. Since this investigation involved the use of a reproducible inorganic medium, one is hardly justified in comparing these results directly with those obtained by investigators using organic media such as various sterile hay or lettuce infusions, to which the bacterial inoculum was added. However, since disagreement already exists among other workers as to the suitability of the food organisms in the organic media, these results do afford a means of accounting for some of these discrepancies.

It has been shown in Table 1 that such species as *B. coli*, *B. subtilis*, and *Ps. fluorescens* have been reported as both suitable and unsuitable food organisms for various species of *Paramecium*. It has been shown here that, in the case of *E. coli*, strains K-11 and K-12 are quite different, the former being the better food. Such was also the case for *B. subtilis*, strain D-39 being far superior to strain D-40 as a food for the ciliate. Somewhat similar observations were made by Hetherington (1934) in his studies on *Colpidium*. He states:

It is an interesting fact that *C. colpoda* is not very particular with regard to the species of bacterium upon which it will grow, although it may be more so than *C. campylum*. Indeed, the particular strain of the species used appears to be quite as important as the nature of the larger group. Thus the *coli* strain from Stanford (*Escherichia coli commune*) and *Aerobacter aerogenes* 5 gave trouble, while *E. communior* 1 and *A. aerogenes* from Stanford were as satisfactory as any bacterium tried.

Thus, upon strain differences within a given bacterial species the disagreement as to the suitability of a given food organism qualitatively standardized may well be explained.

It is also possible to account for the disagreement as to food qualities of a given bacterium in yet another way, since the suitability of *Ps. fluorescens* was found to vary according to the age of the bacteria. Since this species had been reported earlier as both suitable and unsuitable as food for *Paramecium*, strain U-6 of this bacterium was qualitatively standardized at 24 hours and 4 days of cultivation before use. It was found that at the latter age this bacterium served as an adequate control food for all the others tested, since over a period of 6 weeks 5 lines of the ciliate carried in it maintained a composite average daily fission-rate of 1.5. However, when this same strain of *Ps. fluorescens* was used after only 24 hours of cultivation, it proved to be a very unsatisfactory food. Of 5 lines of the ciliates started in the 1-day-old food only 3 were able to survive during the same period as the controls. Although cross-transfer of the paramecia was carried on, the composite average daily fission-rate of all lines started in this 1-day-old food was only 0.2. In comparison with the controls in the 4-day-old food, this very low division-rate indicates that the age of the food organism can be a deciding factor in determining the suitability of a specific food for *Paramecium*.

From this it should be pointed out that, since all the bacteria tested in this study were qualitatively standardized at 24 hours (except for the representatives of the Mycobacteriaceae), some of those which were found to be unsatisfactory may not prove to be so in older cultures.

The degree of suitability of the bacteria was roughly estimated by comparing the fed-animal cultures to one which was subcultured daily into sterile salt solution but without the addition of food. In unfed cultures the paramecia become greatly diminished in size and rather transparent prior to death, which occurs in about 12-14 days when the animal inoculum is 20 per cc. Giese and Taylor (1935) report that *P. multimicronucleata* will die of starvation in Peters' inorganic medium in about 12 days when there are 10 animals per cubic centimeter. Those food organisms which were found to be very unsatisfactory in some cases appeared to cause the death of successive cultures at almost regular intervals, which were much shorter than that required to kill the paramecia by starvation. For example, 7 successive cultures fed *A. lignieresii* died in 4 days of transfer.

In most cases the paramecia died in those foods which have been reported here as poor. However, in suspensions of *M. phlei*, *M. berolinense*, *M. smegmatis*, and *Corynebacterium ovis* the cultures never died out completely. In all these foods the paramecia ap-

peared much like those in the nonfed cultures, indicating that they were probably in a semistarved condition even though there was an abundance of bacteria present. Whether these members of the Mycobacteriaceae are only relatively "toxic" or are lacking in certain food requirements for the ciliate is a matter of conjecture in this ecological study. It is interesting to note, however, that *M. phlei* and *M. smegmatis* have been reported by D. F. Johnson (1936) as unfavorable foods for *Glaucoma ficaria*, cultured also in an inorganic medium. In those food suspensions which have been found here to be very satisfactory the paramecia flourished. This was especially true of those organisms fed *Ps. ovalis*, in which the fission-rate was 1.7. Giese and Taylor (1935) also found *Ps. ovalis* to be a suitable food for *P. multimicronucleata* cultured in a lettuce medium.

Recently Kidder and Stuart (1939) have studied the role of bacteria in the growth and reproduction of *Colpoda* grown in distilled water on 12 species of bacteria in pure culture. Their findings are somewhat similar to those reported here for paramecia. Their *Aerobacter cloacae* was most satisfactory, *Serratia marcescens* much less so, while *Staphylococcus albus* and *Ps. fluorescens* were decidedly unsuitable. Although the 3 species of *Pseudomonas* used were not identified other than by the numbers 81, 82, and 85, one is led to believe that these may be *Ps. fluorescens*. No attempts were made to standardize any of the food organisms.

These investigators did find that distilled-water culture medium will become a nutritive for many bacterial contaminants, as it becomes the solvent for ciliate metabolites and bacterial disintegration products. They found that by transferring the protozoa by means of a bacteriological loop every 48 hours into fresh medium over a period of 6 weeks bacterial contaminants (mostly *Pseudomonas*) so multiplied as to have a deleterious effect upon *Colpoda*.

In this investigation on paramecia such contamination was not observed. At the close of the 4-week period some of the protozoan cultures were tested for contamination. The final 1 cc. of used culture medium in each case was diluted with 9 cc. of sterile water, and these suspensions were examined by plating on nutrient agar. After a period of incubation these test plates showed only isolated colonies of the bacteria characteristic of the food organism used originally as the inoculum. This indicates that if any chance contaminant was present, it had not developed sufficiently to affect the results obtained. It is probable that because of the daily subculture methods the ciliate metabolites and bacterial disintegration products were not present in sufficient quantities to make the salt solution a nutrient for contaminating bacteria. Consequently, it is assumed that the paramecia were feeding on pure cultures of bacteria.

SUMMARY

1. Under standardized methods of culturing *P. multimicronucleata* this organism was found to utilize certain species of bacteria in pure culture as food organisms so that they maintained a high fission-rate over a relatively long period. Other bacteria proved to be less satisfactory foods, and still others were very unsatisfactory. In the latter case such foods caused the death of a given culture in a much shorter time than is usually required to kill a similar culture by starvation.

2. Strains within a given species of bacteria were shown to vary in food qualities as much as different species. This may account for differences previously reported in regard to the suitability of a given species as a food organism for the ciliate.

3. Aging *Ps. fluorescens* 4 days before use so improves its food qualities that it is a

very satisfactory food, whereas if this bacterium is taken from a 24-hour-old slant culture, it is definitely unsuitable.

4. The average daily fission-rate of the protozoa in a specific food suspension was used as a criterion of the suitability of the food organism. In those foods in which the average number of progeny of the ciliate was low this may be accounted for either by the dying-out of successive cultures or by the general unsuitability of the food. In either case the low average fission-rate represents rather clearly the degree of suitability of the food organism. Conversely, a high fission-rate indicates the superiority of the bacterium.

5. From the results of this investigation it is believed that the method of culturing paramecia in an inorganic medium on a pure strain of bacteria under standardized conditions will greatly facilitate experimental studies by insuring reproducible results. The determination of the suitability of various bacteria as possible food organisms under such standardized conditions as were employed should aid in the future selection of foods for *P. multimicronucleata* and should bring about closer agreement in future studies using the isolation-culture method.

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THE EFFECTS OF POPULATION DENSITY UPON GROWTH REPRODUCTION, AND SURVIVAL OF *HYALELLA AZTECA*

(Five figures)

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THE problems of the biotic relationships of like organisms have attracted much interest. Naturalists have reported with enthusiasm noteworthy aggregations, sexual behavior, and "social" manifestations. This work continues but with a more critical attitude. The less obvious mass relations, seen only by resultant effects, led to a dichotomy of interest, which in the future and to some extent at present (Park, 1939) will coalesce. One approach is that of the study of the whole population, its growth, maintenance, and decline or evolution (Chapman, 1928; Wright, 1931; Gause, 1934; Bodenheimer, 1938). The other is the study of physiological or behavioristic effects of population density upon the constituent organisms (Allee, 1931, 1934, 1938).

The physiological aspect is considered in the present study,¹ which attempts a demonstration of the effects of increasing the density of the population upon three fundamental processes: growth, reproduction, and survival. As an assay animal the common freshwater amphipod *Hyalella azteca* Saussure² (*H. knickerbockeri* Bate), Orchestiidae, was chosen. In the use of an aquatic arthropod the study is unique among researches comparable in nature to those of Pearl and of others to be cited later. The conditions of the experiments were variable, but equal volumes of food and water were used in all, irrespective of numbers of animals present. The following brief review of the literature is necessarily closely confined to the question studied and deals with recent studies on arthropods. The references given in the first paragraph include summaries of results on population studies of other forms. Earlier work by the same or different authors may be found by consultation of the literature referred to below.

Growth.—Body size (Warren, 1900) and rate of development (Banta, 1937) were retarded in Cladocera by crowding or its concomitant factors. Metamorphosis in the beetle *Tribolium confusum* was delayed, and body weight was reduced (Park, 1938) in relation to the density of the population and more directly to the degree of conditioning of the medium. On the other hand, evidence is present for an augmentative effect of increasing numbers of organisms. Development is accelerated in *Periplaneta orientalis* after the ninth instar, and the effect is apparent even in a density as low as 2 animals. However, the longest and heaviest animals were found among the isolated individuals, an effect attributed to increased disturbance by collision in higher densities (Landowski, 1938). Meal worms (*Tenebrio molitor*) raised 50 together in 60 cc. of meal consistently weighed more than did isolated larvae; even 150 animals in a similar volume maintained a higher weight than that of isolated ones until the sixtieth day (Michal, 1931). This was

I wish to express my gratitude to my adviser, Dr. W. C. Allee, for his interest in, and criticism of, this problem.

¹ To Mr. Clarence Shoemaker, assistant curator at the Smithsonian Institution, I am indebted for the identification of the animals.

attributed to an effect of increased temperature. Wardzinski (1938) believed this factor effective in the progressive acceleration of larval life in *Pieris brassicae* raised in densities ranging from 1 to 32 animals. The heaviest pupae of this butterfly occurred in populations of 2 or 4. Accelerated development in higher densities was found by Titschack (1936) and Mosebach-Pukowski (1937) in the clothes moth and *Vanessa*, respectively. Landowski (1938) cites Manujlowa, Kosmina, and Alpatov (1931), and Alpatov and Dorodnichaia (1932), as obtaining results indicating the inhibitory action of isolation on lepidopterans.

Reproduction.—Few studies on reproduction in regard to density in arthropods are at hand. The most complete concern *Drosophila melanogaster*, which showed a clearly inverse relationship to numbers of animals present (Pearl, 1932). In cladocerans reproduction was adversely affected by crowding (von Dehn, 1937). The retardative effect of crowding on the group fecundity of scales (*Lepidosaphes ulmi*) was due to the increased number of sterile females rather than to an effect upon the reproducing mothers (Smirnov and Polejaeff, 1934). On the other hand, crowding decreased fecundity in the moths *Arctia caja* and *Lymantria dispar* by lowering the maturation of ovarian eggs. This effect was attributed to malnutrition through disturbance by collision during feeding (Hofmann, 1933).

By counts of the growing populations the reproductive activity of flour beetles seems to be maximal at a population of intermediate density (Park, 1932; MacLagen, 1932; MacLagen and Dunn, 1936). Increased copulation frequency was directly shown to increase fecundity of *Tribolium* (Park, 1933) and may be operative in the greater population development of an initially intermediate density. However, this effect is offset to an increasing extent by the direct retardation of fecundity by increased conditioning of the medium (Park and Woollcott, 1937).

Survival.—A rough generalization on the effects of density upon survival may be attempted: under toxic conditions or those inimical by the absence of essential elements, survival is maximal at an intermediate density since the organisms or their products tend to ameliorate these conditions. Many instances are given by Allee (1931) and, more recently, by Allee and Wilder (1939). Careful analysis of death rate in *Drosophila* populations (Pearl, Miner, and Parker, 1927) demonstrated a similar effect, with the optimal density at 25–35 flies per 1-ounce bottle. However, survival is frequently inverse to the numbers of organisms present. This has been reported for cockroaches (Landowski, 1938) and for larval mortality of grain beetles (MacLagen and Dunn, 1936; Park, 1938).

MATERIAL AND METHODS

Hyaella azteca Saussure (*H. knickerbockeri* Bate) is a widely distributed amphipod. The specimens which were used came from a culture maintained for over ten years in the tanks of the department greenhouse, where they were accidentally seeded.

None of the research done upon amphipods has required the development of the standardized techniques necessary in quantitative studies on physiological effects. The most complete study of amphipods is that initiated by Sexton and continued by herself and others. This chiefly genetic study has been summarized by Sexton and Clark (1936). In their work the animals were kept in finger bowls and fed upon *Ulva* or dead leaves—a method altogether unsatisfactory for results dependent on the control of all factors, as far as possible, other than density of like organisms. In the search for greater control

much of the present work is exploratory and covers a rather wide field of observation in order to lay a basis for further analytical treatment.

The experimental procedure consisted in obtaining the needed number of newly released young within, if possible, 2-3 days. They were then counted out at random into 6- or 8-ounce, narrow-necked bottles, according to the planned population densities. The bottles had been filled with 100 cc. of water, to which were added either 2-inch lengths of *Elodea* or 2-4 drops of yeast. Usually the food and water was changed weekly until the end of the experiment. All experiments were conducted at room temperature, which was recorded by a thermograph (Table 1). Temperatures are given in degrees centigrade.

The above résumé needs further expansion. Several hundred females with brood in about the same stage were collected from the stock and kept in finger bowls. Shortly before the time of expected release of the young, the bowls were cleaned of all young then present; from that time on they were removed at frequent intervals and stored together in finger bowls for use in the experiment.

Elodea was first tried as food because it is one of the natural food plants of the amphipods (Embrey, 1912). The long streamers were washed, stripped of their leaves, and scalded in hot tap water. The latter treatment was planned to kill associated organisms. The food was most unsatisfactory because of inequality along the length of the stem and from stem to stem. The hot-water treatment sometimes killed the plant and did not always kill the animals on the plant. For these reasons *Elodea* was abandoned after the third experiment.

Yeast was substituted, since it is a simple, easily measured food. Fleischman's yeast cake was made up with distilled water in the proportion of 1 gm. to 10 cc. The well-stirred stock suspension was fed to the populations by drop. The yeast in the population bottles was not itself growing but started to grow upon removal to a suitable nutrient solution. This took place even after the yeast had been 8 days in the clean water medium. No attempt at sterile precautions or at bacterial analyses has been made.

The initial densities seeded changed with the deaths of some of the animals. In order to maintain, as nearly as possible, the original densities, one or more bottles of a given population were eliminated, and the animals used to restore the numbers in the remaining bottles of that density. This could not be done in densities of 20, 50, and 100, where few bottles were available, especially in the two highest densities. Here the densities were allowed to drop but never to overlap. These populations were equalized by transference of animals between bottles of a given density.

The molted skins (exuviae) were collected at each time of change of water, or oftener, and preserved in 50 per cent alcohol. Later, magnified projection outlines of the mid-dorsal lines were drawn, and these were measured by calipers.³ It is necessary to use the exuviae for an accurate total-length measure because the animals are flexible, and the segments telescope within each other. The living whole animals, even when anesthetized, displayed all degrees of flexion. Since the exuviae were transparent, the overlap of segments might be seen, and the drawings were made to include this factor. The total lengths given, therefore, are those of the summated sclerites. The figures are the most accurate possible, but they are entirely dependent on a rough sampling of the population. As the animals became older, the validity of the representation suffered greatly because they molted less often, and they destroyed the exuviae. The latter was particularly

³ I wish to thank Miss Catherine Lutherman for her assistance in these measurements.

TABLE 1
EXPERIMENTAL CONDITIONS

Expt. Number	Initial Density	Number of Bottles	Remarks
<i>Elodea</i> I.....	<div> <div>2</div> <div>5</div> <div>10</div> <div>25</div> <div>50</div> <div>100</div> </div>	<div>25</div> <div>10</div> <div>5</div> <div>4</div> <div>2</div> <div>1</div>	Started 12/7/37, mean age of young: 2 days; temperature: mean 19°3, range 16°0-23°0; food and water: <i>Elodea</i> , tap water, both changed every other week; total number animals seeded: 450
<i>Elodea</i> II.....	<div> <div>1</div> <div>10</div> <div>20</div> <div>50</div> </div>	<div>50</div> <div>5</div> <div>2</div> <div>1</div>	Started 1/16/38, mean age of young: 2 days; temperature: mean 19°4, range 16°0-24°0; food and water: <i>Elodea</i> (living), charcoal-filtered tap water changed weekly; total number animals seeded: 190
<i>Elodea</i> III.....	<div> <div>1</div> <div>8</div> <div>20</div> <div>50</div> </div>	<div>50</div> <div>5</div> <div>2</div> <div>1</div>	Started 1/16/38, mean age of young: 6 days; temperature: mean 19°4, range 16°0-24°0; food and water: <i>Elodea</i> (living), charcoal-filtered tap water, never changed, poured through silk bolting cloth weekly; total number animals seeded: 180
Yeast I.....	<div> <div>1</div> <div>2</div> <div>4</div> <div>10</div> <div>20</div> <div>50</div> <div>100</div> </div>	<div>50</div> <div>50</div> <div>25</div> <div>20</div> <div>5</div> <div>2</div> <div>1</div>	Started 5/3/38, mean age of young: 1 day; temperature: mean 25°2, range 21°0-30°0; food and water: 2 drops yeast to 6/14, then 4 drops, filtered tap water, both changed weekly; total number animals seeded: 750
Yeast II.....	<div> <div>1</div> <div>2</div> <div>4</div> <div>6</div> <div>10</div> <div>20</div> </div>	<div>100</div> <div>50</div> <div>25</div> <div>20</div> <div>20</div> <div>10</div>	Started 8/3/38, mean age of young: 2 days; temperature: mean 28°0, range 24°0-31°3; food and water: 4 drops of yeast, filtered tap water, both changed weekly; total number animals seeded: 820
Yeast III.....	<div> <div>1</div> <div>2</div> <div>4</div> <div>6</div> <div>10</div> <div>20</div> </div>	<div>100</div> <div>60</div> <div>25</div> <div>20</div> <div>10</div> <div>5</div>	Started 12/7/39, mean age of young: 2 days; temperature: mean 21°0, range 19°5-22°0; food and water: 4 drops yeast, filtered tap water, both changed weekly; chlore-tone used after second week; total number animals seeded: 640; replacements on fourth day
Yeast IV.....	<div> <div>1</div> <div>2</div> <div>4</div> <div>6</div> <div>10</div> <div>20</div> </div>	<div>60</div> <div>30</div> <div>15</div> <div>10</div> <div>6</div> <div>3</div>	Started 2/22/39, mean age of young: 4 days; temperature: mean 20°9, range 19°5-22°0; food and water: 4 drops yeast, well water, both changed weekly; chlore-tone used after second week; total number animals seeded: 420
Yeast V.....	<div> <div>1</div> <div>2</div> <div>4</div> <div>6</div> <div>10</div> <div>20</div> <div>50</div> </div>	<div>100</div> <div>50</div> <div>25</div> <div>20</div> <div>20</div> <div>10</div> <div>4</div>	Started 2/26/39, mean age of young: 3 days; temperature: mean 20°9, range 18°6-24°0; food and water: 4 drops yeast, supplemented after 1 month of age by 2 inches <i>Elodea</i> stem, well water, both changed weekly; chlore-tone used after one month; total number animals seeded: 1,020
Yeast VI.....	<div> <div>1</div> <div>2</div> <div>4</div> <div>6</div> <div>10</div> <div>20</div> </div>	<div>80</div> <div>40</div> <div>20</div> <div>14</div> <div>8</div> <div>4</div>	Started 5/10/39, mean age of young: 4 days; temperature: mean 24°9, range 19°2-30°0; food and water: 4 drops yeast, well water, both changed weekly; chlore-tone used in first week; total number animals seeded: 564

true in a density of 10 or more. The measurement of size by exuviae has been used in a study of proportional growth in *Gammarus* (Sexton, 1924).

A second method was used after experiment Yeast II. All animals of a population were anesthetized in chloretone, and the head length was measured by ocular micrometer. The increased deaths from this method invalidated the survival record. However, the error in the growth analysis due to partial sampling was entirely obviated. To substantiate the results obtained in growth by measurements of body or head lengths the sum of the antennal segments of the first and second antennae of one side was usually recorded also ("antennal segment number").

Fecundity was demonstrated by removal and preservation of the females carrying brood. They were later measured, and the eggs stripped from the pouch and counted. Only three experiments lasted long enough with a sufficiently high survival to provide such data.

By counting the animals when the water was changed, the number surviving was easily obtained. Survival has been followed only until the time of female maturity, to avoid the necessity of a correction factor for the removed females. Also survival records have not been used after anesthesia by chloretone.

EXPERIMENTAL RESULTS

GROWTH

Isolated controls.—The broods of 10 females were isolated, on May 4, 1938, 1 young per 100 cc. of filtered lake water with 1 drop of yeast. Initially, 107 first-instar animals were obtained. The water and food was changed weekly; at the thirty-eighth day the

TABLE 2*

GROWTH OF ISOLATED *H. azteca* FED UPON YEAST FROM THE FIRST TO THE TENTH INSTAR

Instar	Days of Age at Given Molt	Duration of Instar (Days)	Body Length (Mm.)	Increments of Growth (Mm.)	Percentage Head to Total Length	Number of Antennal Segments of One Side
1.....	5.2±0.1	5.2±0.1	1.28±0.01	13.6	13.0±0.1
2.....	10.7±0.2	5.4±0.3	1.53±0.02	0.24±0.02	12.5	13.6±0.1
3.....	16.6±0.3	5.9±0.2	1.83±0.03	0.31±0.02	12.0	14.4±0.2
4.....	22.6±0.4	6.1±0.3	2.17±0.04	0.33±0.03	11.2	15.2±0.2
5.....	28.9±0.4	6.1±0.3	2.48±0.05	0.31±0.03	10.9	16.6±0.2
6.....	34.5±0.6	5.9±0.3	2.83±0.04	0.35±0.02	10.4	17.6±0.2
7.....	41.9±1.1	7.6±0.3	3.23±0.06	0.38±0.02	10.1	18.9±0.3
8.....	49.6±1.2	8.8±0.4	3.65±0.06	0.42±0.02	10.0	19.9±0.3
9.....	58.0±0.7	9.3±0.4	4.00±0.08	0.38±0.03	9.5	21.1±0.3
10.....	65.2±1.2	9.1±0.4	4.40±0.11	0.37±0.04	9.4	22.1±0.7

* Means and standard errors are calculated from 72 animals providing data on time and age, of which 42 were used in the calculation of morphological values.

food was increased to 2 drops of yeast, and on the seventy-second day to 4 drops. This experiment was run synchronously with Yeast I; therefore the conditions are comparable to those of that experiment. The bottles were observed daily, molts recorded, and exuviae preserved for later measurement. Only the data pertinent to this problem will be discussed. Deaths reduced the number of animals to 73, and of these only 42 series of exuviae were sufficiently complete for growth records. Table 2 presents the mean values and their standard errors.

TABLE 3
MEAN BODY LENGTH (MM.) OF ANIMALS IN POPULATIONS
FED UPON *Elodea*

(The lower half of each table presents the significance [*P*]
of the difference between the designated populations)

A. *Elodea* I

AGE IN DAYS	INITIAL POPULATION DENSITY					
	2	5	10	25	50	100
16.....	2.02	2.10	2.19	2.18	2.11	1.84
40.....	4.27	3.75	3.70	3.83	3.33	2.51
45.....	5.32	4.70	4.48	4.25	3.72	2.90
52.....	5.79	5.64	5.41	4.47	3.78	2.95

AGE IN DAYS	COMPARED POPULATION DENSITIES					
	2:5	5:10	10:25	25:50	50:100	2:10
16.....	0.44*	0.33	0.84	0.16	0.008	0.11
40.....	0.04	0.22	0.02	0.001	0.0001
45.....	0.03	0.38	0.27	0.02	0.002
52.....	0.63	0.49	0.01	0.06	0.010

B. *Elodea* II

AGE IN DAYS	INITIAL POPULATION DENSITY			
	1	10	20	50
9.....	1.39	1.38	1.34	1.23
16.....	1.91	1.76	1.89	1.72
23.....	2.32	2.44	2.25	1.87
30.....	3.16	2.97
72†.....	5.18	4.27	3.69	3.04

AGE IN DAYS	COMPARED POPULATION DENSITIES		
	1:10	10:20	20:50
9.....	0.92	0.42	0.11
16.....	0.13	0.13	0.06
23.....	0.62	0.39	0.12
30.....	0.43
72.....	0.0001	0.09	0.15

* The values are probabilities (*P*). A *P* of 0.05 (5 chances in 100 of getting a similar difference by "chance") is regarded as the upper limit of significance. The smaller the decimal the greater is the significance.

† Lengths are of all survivors. Sexes approximately equally represented.

The changes from instar to instar were quantitative; no qualitative criteria were found. The antennal segment number for each instar was highly variable, and there are indications that it cannot be regarded as a valid index of instar. Sexual differentiation also was gradual. No earlier than the seventh instar (about 40 days), often much later, the males might be distinguished by the heterogonic development of the large second gnathopods. On the average, the males in the experiments were larger after maturity than were the females. In the experimental results presented later the percentage of developed males is given, when possible, to indicate whether disproportion of the sexes among the populations is correlated with mean size differential. The first molt occurred at about 5 days of age (Table 2). In the experiments body length was first measured at least 1 week after the start of the experiment, at which time the animals would usually be between 8 and 11 days of age. Therefore, the first recorded mean size covers a mixture of first- and second-instar animals. Table 2 shows also the percentage relation of head to total length, since these two measures have been utilized in the experimental results.

TABLE 4*
GROWTH IN POPULATIONS FED UPON YEAST IN EXPERIMENTS I-VI

AGE IN DAYS	INITIAL POPULATION DENSITY					
	1	2	4	6	10	20
10.4.....	1.67	1.73	1.76	1.78	1.77	1.78
17.6.....	2.41	2.47	2.55	2.52	2.43	2.56
26.2.....	2.96	3.00	3.02	2.98	2.84	2.84
33.7.....	3.83	3.96	3.89	3.56	3.44	3.32
44.0.....	4.51	4.29	4.41	4.23	4.13	3.95

* The values are means of total length and head length $\times 10$ (mm.). The boldface figures are maximal for each age.

Growth of animals in populations fed on Elodea.—Of the three experiments in which *Elodea* alone was used as food, only two furnished data upon the growth of the animals. In the third experiment, in which the water was never changed, insufficient numbers of molted skins were obtained, either because the animals molted rarely or at once destroyed them. From Table 3 it may be seen that, on the whole, mean body size was inversely related to the density of the population. The maximal size at density 10—16 days—in *Elodea* I was not significant, but the indication is of interest in view of later results. The significance of the difference between densities increased with age (Table 3, A, 16 days compared to 40 days) and with increasing density (25:50, compared to 50:100). In *Elodea* II none of the observed differences is significant until age 72 days, at which time the isolated individuals were significantly larger than those in population 10.

Growth of animals in populations fed upon yeast.—When compared to the results of *Elodea* feeding, animals in populations fed upon yeast show another effect. Table 4 presents an average of the six experiments at the ages and densities which are comparable. Population density 6 has been interpolated for Yeast I. The results on head length in Yeast III through VI have been multiplied by 10 to weight them nearly equal to body lengths. The table shows a shift of mean maximum size from a high density to lower den-

sities with advancing age. The results of separate experiments bring this point out more clearly. Figure 1 presents the results of the first yeast experiment. In this experiment only 2 drops of the stock yeast suspension were added to the 100 cc. of filtered lake water. After the experiment was in progress, it became apparent that this was not sufficient, since population 10 cleaned it out of the water before the week between feedings was complete. However, such feeding was continued until the maximal growth of population

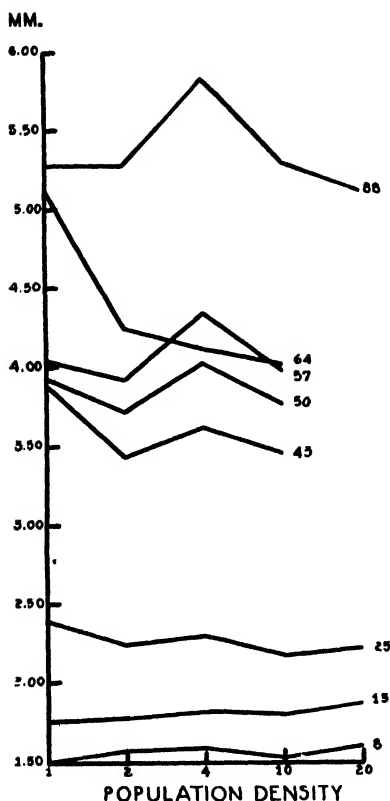


FIG. 1.—Mean body length of populations in Yeast I. Age in days to the right of each curve.

1 was well established (Fig. 1, 43 days). On the forty-fifth day bi-weekly feeding was started, making a total of 4 drops per week to each population, an amount used in all later experiments. Only the densities through 20 have been used in the figure. Density 100 provided practically no exuviae for length measurements; the density of 50 and 20 gave data through the twenty-fifth day. The failure of data for these higher densities is largely due to the destruction of the exuviae and, possibly, to less frequent molting. Consequently, the few molted skins obtained were an unreliable sample. However, the trend of the higher densities is indicated at the eighty-eighth day by the result in density 10 and 20. At this age all remaining males were preserved and later measured so that the values are derived from the whole male population. The populations did not invariably fit the trend. Pearl and Parker (1922) write that, owing to accidents relative to the smallness of numbers at certain populations, their populations did not "fall in line." The discussion of survival will show this more clearly in the amphipod populations (Fig. 5).

During the first 2 weeks of age the animals of population 20 were significantly larger than those isolated ($P = 0.017$ and 0.002 for 8 and 15 days of age). Thereafter, body length tended to become inverse to the population density, although the difference between densities of 1 and 2 was not significant. After the food was doubled in quantity, an insignificant ($P = 0.271$) maximum occurred at density 4 through the fifty-seventh day. Failure of significance is related to the small number of exuviae obtained in any population and their great variability in length. At the sixty-fourth day the isolated animals were on the average of greatest body length. The difference between densities 1 and 4 is significant ($P = 0.028$), despite the fact that the 24 isolated animals for that day were represented by 6 exuviae only. The sampled animals of population 4 were of smaller size in general than on the preceding date (57 days). Probably those molted skins were from females, which at sexual maturity show the inverse relationship of body length to density (Fig. 4, C) and which at that time, in general, are smaller than the males. Males, on the

other hand, in the three experiments (Yeast I, IV, V) which continued long enough for this measure, invariably were of greatest mean length in a density of 4 (Fig. 3). The latter observation was shown in Yeast I by the mean body lengths of the preserved males at the eighty-eighth day of age. This curve strongly suggests those of the three ages prior to the sixty-fourth day. Counts of the developed males made on the sixty-eighth day showed the highest proportion of that sex in the population density of 4; the percentages for populations 1-50 were, respectively, 41.2, 54.0, 64.9, 51.2, 48.0, and 14.6.

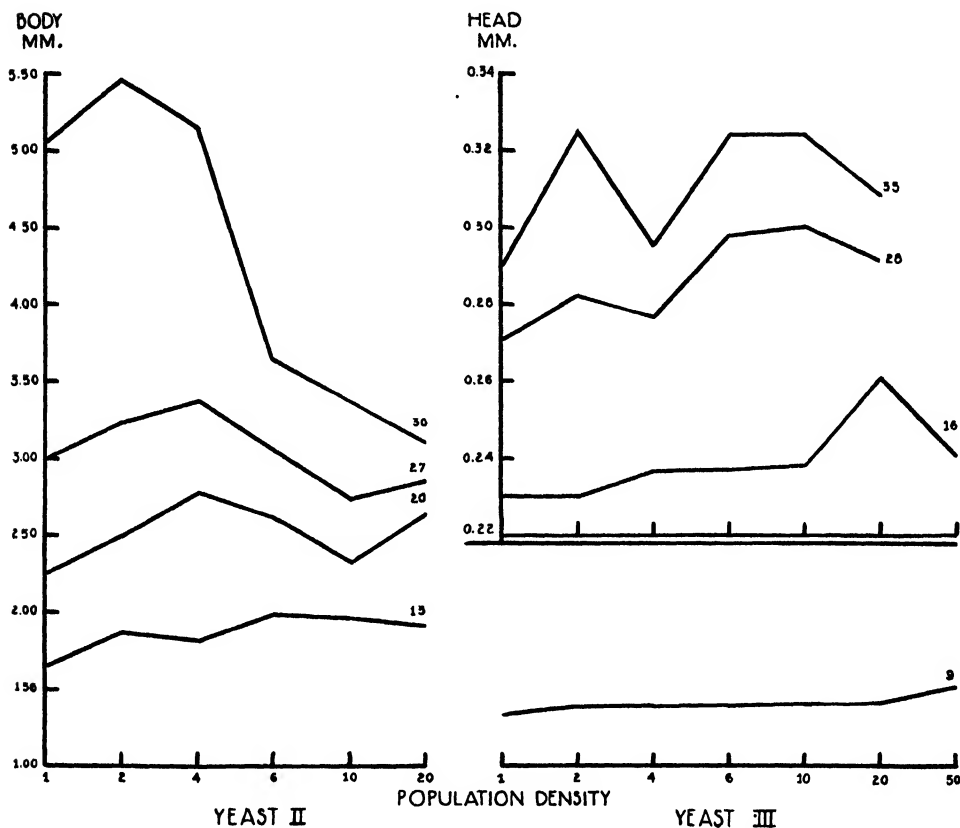


FIG. 2.—Mean body or head length of populations fed upon yeast. Age in days to the right of each curve

These figures are not basic sex ratios, which are approximately 1:1, because the females which had shed their eggs had been removed from the population and because the immature males are practically indistinguishable from females without brood or mature ovaries. There is, unquestionably, delay of sexual maturity in populations of 50 or more. The percentages given above indicate that the preponderance of developed males may have occasioned the peak of size at population 4 from the forty-third day.

The next two experiments were planned to demonstrate the growth of the animals in the populations prior to sexual maturity, in order to obviate the complication introduced by the presence of mature males and females. Both are presented in Figure 2. The sec-

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ond type of length measure was instituted in the second week of Yeast III (16 days of age); all animals were anesthetized, and the head lengths measured by an ocular micrometer. The values are, therefore, means of the entire population. The same is true of the thirtieth day in Yeast II, for the values are derived from measures of the whole preserved animals. The results show a shift of mean maximal size from a higher density to a lower with advancing age. In Yeast I (Fig. 1) the isolated animals were maximal in size on the twenty-fifth day; with double the food the shift in Yeast II and Yeast III had fallen to the density of 2 at the thirtieth and thirty-fifth days, respectively. The delayed drop of the maximum may be correlated with the increased quantity of food. Yeast III started on the ninth day with maximal exuvial length in population 50, but in Yeast II on the thirteenth day the population 6 is the largest. The difference between the two experiments is not explainable by changes in technique, but it does not seem unreasonable, since the former was started in December and the latter in August.

In Yeast II the population of mean maximal size was at no time significantly bigger than the isolated animals. However, in Yeast III significance obtained at all but the last age; the P values, the maximum compared with the isolated animals at the four ages, are, respectively, 0.003, 0.0001, 0.016, and 0.093.

The preceding three experiments have given reasonably comparable results. The water used in all three was charcoal-filtered Lake Michigan (city) water from the laboratory tap. In order to see whether the drift of maximal growth was related to the type of water used, a change was made in the next three experiments (Yeast IV, V, VI) to Whitman well water. Quantity of food was maintained as in Yeast II and Yeast III at 4 drops per week.

Yeast IV has been chosen to be presented in complete tabular form (Table 5), since this experiment included exuvial length, head length, and antennal segment number through the sixtieth day.

The outstanding feature of Yeast II and Yeast III—namely, the shift of mean maximal length from a high density to a lower—occurred again, although somewhat less regularly. With great individual variation, as has been previously mentioned, sexual maturity starts after the fifth week, so that the maximal size of density 4, from the thirty-ninth day, as in Yeast I, may be influenced by the presence of the males. As before, the percentage of developed males was greatest among the animals of density 4. These percentages are 41.6, 35.7, 46.1, 34.8, 41.2, and 35.0 for population densities 1–20, respectively. Also the males of density 4 are largest, although not significantly (Table 5, 60 days; Fig. 3).

The absence of exuvial values for populations 2 and 10 on the thirty-second day of age is due to the fact that no molted skins represented those populations. The destruction of "molts" throughout all populations was so great thereafter that collection of them was abandoned. The data on head length, by their freedom from sampling error, are more reliable, but both measurements show in part a significantly greater size of the animals in the higher densities at an early age. The animals represented on a given date by molts were of an earlier instar than the same animals within the whole population valued by head length on the same date. In C with exceptions the number of antennal segments tends to support the growth effect presented in A on the basis of length.

Yeast V was designed originally for the study of survival and fecundity only. It was very difficult to see the eggs within the brood pouch in yeast-fed females because the yolk remains practically colorless. The addition of *Elodea* to the 4 drops of yeast after

TABLE 5

GROWTH OF ANIMALS IN YEAST IV

A. MEAN BODY (a) AND HEAD (b) LENGTHS IN MILLIMETERS

AGE IN DAYS	INITIAL POPULATION DENSITY					
	1	2	4	6	10	20
11 a*	1.56	1.50	1.67	1.65	1.67	1.66
18 a	1.96	1.93	1.97	1.84	1.86	2.04
b†	0.27	0.27	0.28	0.28	0.28	0.28
25 a	2.43	2.45	2.54	2.43	2.42	2.43
b	0.31	0.31	0.32	0.31	0.30	0.31
32 a	2.98	3.26	2.89	2.90
b	0.36	0.38	0.37	0.35	0.34	0.34
39 b	0.40	0.41	0.43	0.39	0.39	0.38
46 b	0.45	0.44	0.46	0.43	0.44	0.40
53 b	0.48	0.47	0.48	0.45	0.47	0.46
60 b†	0.51	0.52	0.53	0.52	0.51	0.46

B. SIGNIFICANCE (P) OF DIFFERENCE BETWEEN ABOVE MEAN LENGTHS

AGE IN DAYS	COMPARED POPULATION DENSITIES								
	1:2	2:4	4:6	6:10	10:20	1:4	1:6	1:10	1:20
11 a.....	0.42	0.016	0.37	0.62	0.92	0.004	0.021	0.021	0.004
18 a.....	0.69	0.37	0.004	0.48	0.009	0.84	0.09	0.23	0.33
b.....	0.84	0.09	0.92	0.32	0.37	0.02	0.036
25 a.....	0.84	0.48	0.19	0.76	0.92	0.32
b.....	0.48	0.19	0.13	0.11	0.48
32 a.....	0.13	0.23
b.....	0.028	0.62	0.06	0.27	0.55
39 b.....	0.48	0.31	0.009	0.48	0.32	0.09
46 b.....	0.84	0.42	0.07	0.32	0.0001	0.61
53 b.....	0.56	0.55	0.19	0.48	0.84
60 b.....	0.62	0.84	0.69	0.42	0.001	0.54

C. ANTENNAL SEGMENT NUMBERS

AGE IN DAYS	INITIAL POPULATION DENSITY					
	1	2	4	6	10	20
11 a*	13.0	13.0	13.0	13.0	13.0	13.1
18 a	14.8	14.3	14.6	14.6	14.6	14.8
b†	15.7	15.4	15.9	15.7	15.5	15.7
25 a	15.7	16.1	16.3	16.2	15.4	16.3
b	16.9	17.1	17.2	17.0	17.0	17.2
32 a	19.0	18.6	16.8	17.6
b	19.3	19.5	19.4	18.4	18.3	17.9
39 b	20.4	21.1	21.2	20.4	19.8	19.4
46 b	22.2	22.1	22.2	22.7	21.3	20.3
53 b	23.4	22.7	22.8	21.9	22.1	21.1
60 b†	23.8	23.2	24.4	22.3	22.8	21.6

* a = body length or antennal segment number derived from exuviae.

† b = head length or antennal segment number from anesthetized animals, except at 60 days when preserved males used.

‡ Each population represented only by males.

the first month was planned to obtain green-colored eggs. It was hoped that by starting a large experiment, not using chloretoone, and feeding in this way, a great number of fecund females would be obtained. After 1 month of age the survival was so high that it was decided to risk the deaths by chloretoone and take a measure of growth. The readings after the first were of animals fed yeast and *Elodea*.

At the age of 34 days the animals in density 4 were of significantly ($P = 0.028$) longer average head length (0.38 mm.) than those in density 2 (0.36 mm.), which did not differ significantly ($P = 0.76$) from isolated animals (0.36 mm.). The higher populations showed a drop progressively, starting with 6 (0.36 mm.). At 48 days of age, 14 days after the first *Elodea* feeding, the isolated animals were of insignificantly maximal size ($P = 0.33$ when compared with density 2). As before, the males of density 4, on the sixty-second and seventy-sixth day were maximal in head length and antennal number

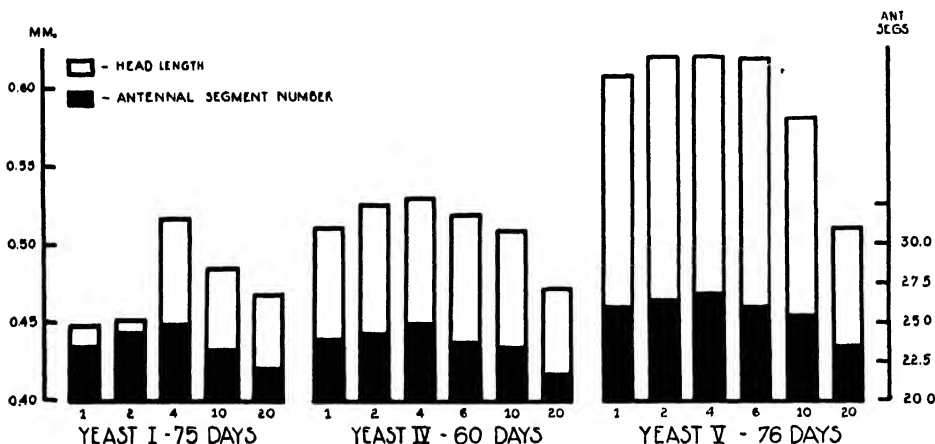


FIG. 3.—Head lengths (left ordinates) and antennal segment numbers (right ordinates) of sexually mature males fed upon yeast. Abscissae, population density.

(Fig. 3). This difference was not significant ($P = 0.19$). The results on mature males in Yeast I, IV, and V are presented in Figure 3. The head lengths of these males are not significantly greater than those of sparser populations except in Yeast I, where the mean head size of density 4 has a P value of 0.007 over that of density 2. The body length of sexually mature females, on the other hand, is inverse to density at the mean age of first oviposition (Fig. 4, C).

Yeast VI was started at the same time as Yeast V and was designed purely to recheck the growth effect. Since no survival data were required, chloretoone anesthesia was begun at the end of the first week (11 days of age). Either for this reason or for some other, the results were atypical in comparison with the other yeast experiments. At 11 days of age maximal head size was obtained in the density of 4, but thereafter the mean greatest head length was displayed by 2, 1 and 2, 2, and 4 in that order in the five determinations covering the 39 days of the experiment. Neither do the antennal segment numbers correlate well with head size. None of the maxima is significantly greater than the mean value from sparser populations.

When measurements were made on the eleventh day one-half of the animals (40)

were anesthetized and measured. It would have been impossible to measure 80 animals in 6 densities within one day, hence the number was halved. This may have contributed to the atypical results, but two technically interesting facts emerged. The deaths were much greater the week after the use of chloretone on those animals, 18.1 per cent as compared to 8.0 ($P = 0.001$), and they were of smaller mean head size; the difference of 0.015 mm. is significant ($P = 0.0004$).

In conclusion, from the results on growth in relation to the density of the population two effects have emerged. When the animals were fed upon *Elodea*, growth was inversely related to the density of the population in city water with and without chlorine. In the former case a slight indication of a maximum in a density of 10 was obtained at the sixteenth day, but this was not significant. However, when yeast was used as food and the water was either dechlorinated city water or well water, early growth was greatest at a high density; but with increasing age animals in lower densities were, on the average, larger. This was a fairly regular shift. The above trend recurred in five experiments, and frequently a statistically significant difference was observed. This was especially true when the compared densities were several steps apart in the experimental gradation. There is an indication that increased quantity of yeast will shift the maximum toward higher densities. The mature males of mean greatest size occurred without exception in the population density of 4. The isolated mature females, on the other hand, at the average age of shedding of first eggs were of largest size.

REPRODUCTION

Behavior.—A mature female oviposits about every 8 days. The eggs are incubated for about 6 days, and shortly thereafter the hatched young leave the pouch. Toward the end of incubation a male carries the female by hooking the dactyl of the first (small) gnathopods under the lateral edges of the second thoracic segment. Amplexus may last only 1 day prior to oviposition, or it may continue much longer if the female is skipping one or more ovulations; a duration of 28 days continuous amplexus has been observed. However, it apparently never occurs during the first 3 days of incubation. On the average it may be said that the male carries the female for about 4 days. During this time release of the brood of the previous mating, molting of the female, insemination, and oviposition occur.

In *H. azteca* the male does not necessarily desert the female at molting. In fact, he may apparently assist the molt (Sexton and Matthews, 1913) by arching up over the female's back and scraping the integument with the uropods. Afterward, insemination occurs during a frequently repeated palpation of the base of the pouch. During this action the male shifts his grasp on the female in order to lie diagonally crosswise and to curve his abdomen beneath that of the female. Oviposition usually takes place within 12 hours of molting, and further attempts at copulation are vigorously resisted, although the male continues for some hours to carry the female.

Female amphipods of many kinds are said never to shed eggs unless carried at some time in the cycle by a male. Embury (1912) cites this to be the case for all amphipods which he studied, including *H. knickerbockeri* (a synonym of *H. azteca*). However, in the present experiments females isolated from the first instar shed eggs. Sexton (1924) reports failure of shedding eggs by isolated females of *Gammarus chevreuxi* and *G. pulex*, but not in *G. locusta*. There were, however, indications of a larger brood size in the latter females when mated. The sterile eggs do not develop and are cast within a few days.

While compiling data upon life-history, counts of the broods of 346 stock females were made at various times. On the average 5.2 eggs were produced per stock female. Two tests of the correlation between brood size and female size (using head length as an index) were made. Forty-six animals measured on July 12, 1937, showed an average head length of 0.510 mm. and a brood size of 4.5 eggs. The correlation is significant ($r = 0.627 \pm 0.089$).⁴ Seventy-four others (July, 1939) gave 0.493 mm. head length and 4.0 eggs per brood ($r = 0.537 \pm 0.083$). Since amphipods increase in size at each molt, even after sexual maturity, it is impossible to state with these measurements whether the correlation is more directly related to female size or to female age.

An increase in fecundity with increasing age, presumably up to a certain limit, has been reported for amphipods (O'Brien and Yarnold, 1937; Embury, 1912; Sexton and Matthews, 1913). However, these authors did not attempt to segregate the factors of age and body size.

The effect of density.—Two hundred and sixty-eight females from three experiments provided the data to be discussed and represent three conditions of food: *Elodea* (Elodea I); yeast supplemented after 1 month by *Elodea* (Yeast V); and yeast alone (Yeast I). The results are graphically presented in Figure 4. In *Elodea* I densities 5 and 25 have been plotted with 4 and 20. Density 100 is omitted from the curve of *Elodea* I because at the time of last observation (70 days) only 2 females had shed eggs. Omitted also are densities 100 and 50 from Yeast I, since no matured females represented the first and only one the second at the seventy-seventh or last day of record. Otherwise, all densities of the three experiments are represented by from 5 to 30 females. The higher numbers occurred in densities 10–25, probably correlated with the higher survival (Fig. 5). The lack of females from densities 100 and 50, however, was not associated with a low survival but resulted from an actual retardation or inhibition of maturity. This is presaged by the retarded age at sexual maturity of the animals in density 20 and also in density 50 where represented (Fig. 4, B).

By analysis of the combined data from populations 2, 4, 10, and 20, which are densities present in all three experiments, a few observations pertinent to the relation of reproduction and diet may be drawn. The differences mentioned below are highly significant ($P < 0.0001$), unless otherwise stated. The sexually active females fed upon *Elodea* throughout life were larger (5.62 mm.) than those fed upon yeast for all (4.56 mm.) or for the first month of life (4.47 mm.). The addition of *Elodea* after 1 month had no significant effect on body size when these animals are compared with those fed upon yeast continually ($P = 0.19$).

The fecundity of the *Elodea*-fed females (8.9 eggs per brood) was much higher than that of the yeast-fed (4.7 eggs per brood). The addition of *Elodea* to a yeast diet significantly increased fecundity (5.9 eggs, $P = 0.005$), but the fecundity from the constant *Elodea* diet was even higher ($P = 0.0001$).

As the yeast-fed animals were retarded in body length and fecundity, so also were they retarded in age at the shedding of the first eggs (60.7 days old) compared to that of the *Elodea*-fed animals (52.9 days). The most rapid development occurred, however, among the animals fed upon both (47.7 days).

In such comparisons it is necessary to consider temperature. The maximal response of the *Elodea*-fed females occurred in a midwinter experiment (19° 3 average temperature), the retarded yeast-fed animals were living under a mean temperature of 25° 2 in

⁴ An r value which is greater than twice its standard error has statistical significance.

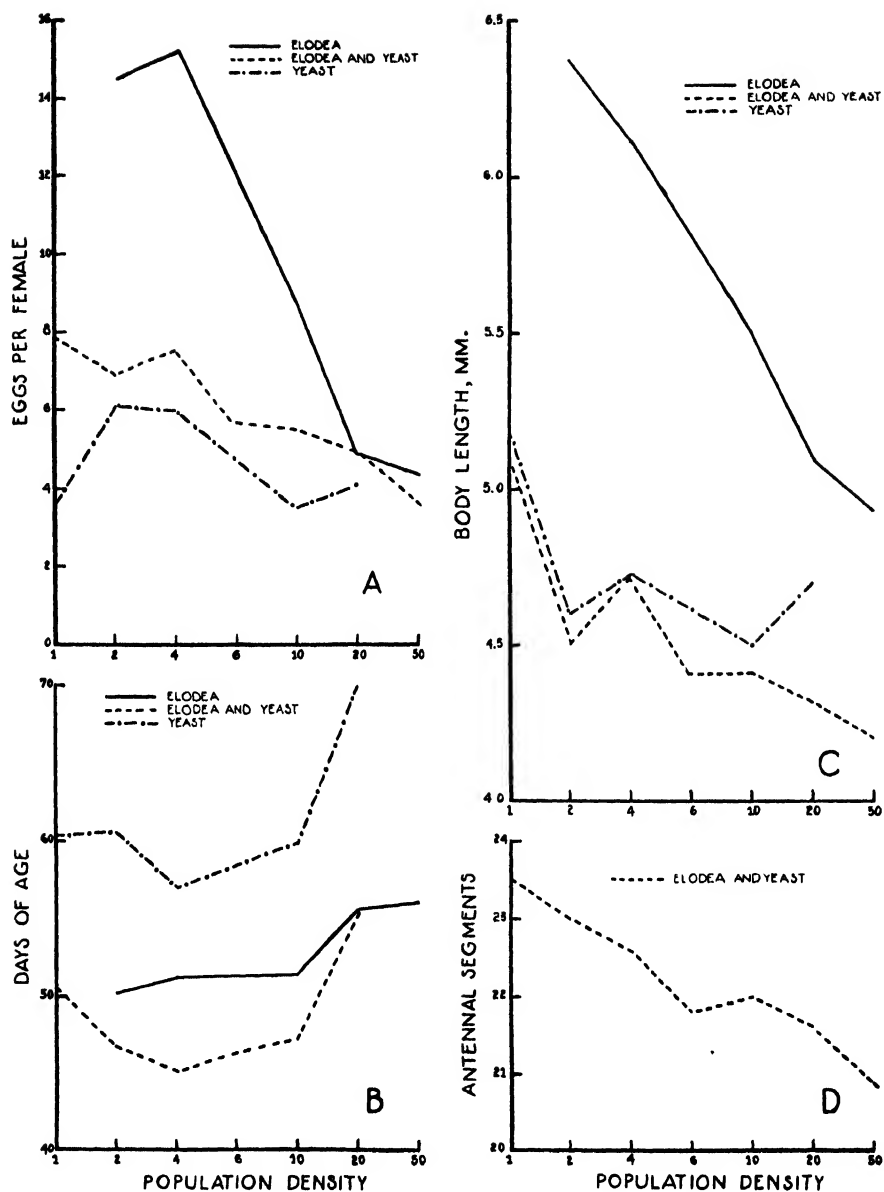


FIG. 4.—Mean fecundity (A), age at first oviposition (B), body length (C), and antennal segment numbers (D) of females in Elodea I (—), Yeast V (---), and Yeast I (- · -).

midsummer, and the yeast and *Elodea* feeding was conducted in the spring with an average temperature of 20°9 (Table 1). Therefore, the difference in rate of development in respect to *Elodea* and to yeast is not in accord with the usual temperature effect. This effect was seen in stock females, of which the summer animals had an interovulatory period of 7.2 days (July) and in winter 12.2 days (January). Temperature may, however, have been effective in inducing significantly more rapid sexual development in the yeast-fed and *Elodea*-fed animals compared to that under constant *Elodea* feeding.

In relation to the effect of density upon reproductive activity it was found that the mean body size of females at the time of first oviposition was roughly inverse to the density (Fig. 4, C). The *Elodea*-fed animals showed the trend most clearly, but even on a yeast diet the difference between isolated and paired animals was significant ($P=0.024$). The antennal segment number, taken only in the yeast-*Elodea* experiment, was also inverse to density (Fig. 4, D). If antennal segment number is an index to instar, it is necessary to say that the 24 females in a density of 50 reached sexual activity at an earlier instar than the isolated animals, for the former possess on the average 20.9 segments and the latter 23.5. By reference to Table 2, the observed 20.9 segments would correspond to about instar 9, and the higher figure to instar 11 or 12. This result throws grave doubt on the validity of correlating antennal segments and instar stage, since a density as high as 50 animals, at least at the age of sexual maturity, has retarded all processes here studied.

Age at first sexual activity (Fig. 4, B) showed a tendency to increase with increasing density when the animals are fed upon *Elodea*. However, in the experiments in which yeast was used, sexual maturity on the average occurred most rapidly in population 4, the same density in which males had the largest size (Fig. 3). The accelerated maturity of density 4 over density 1 is significant in Yeast V ($P=0.003$).

The relation of fecundity to density is made difficult by the absence of a density 1 in *Elodea* I and the paucity of isolated females in Yeast I. In the latter experiment, however, the 12 paired females shed significantly more eggs than did the 5 isolated (Fig. 4, A; $P=0.010$). This is contrary to the correlation in stock females between body and brood size, for these more fecund paired females were smaller in size than the less fecund isolated animals. In *Elodea* I the increase in brood size of density 4 as compared to density 2 is insignificant ($P=0.84$), but the drop from density 4 to density 10 approaches significance ($P=0.09$). In consideration of the three curves (Fig. 4, A), with an increase in density above 4, an inverse relationship appeared between fecundity and density. Prior to that density the numbers present seemed to have little effect, at least when the natural food *Elodea* is included in the diet.

In conclusion of the section on reproductive activity it may be said that *Elodea* fed constantly from birth increased the size of the females when adult, the fecundity, and the rate of sexual development as compared to the effect of a yeast diet. The latter two aspects were also accelerated when *Elodea* was added to a yeast diet.

The females showed in general that body length and antennal segment number were in inverse relation to density. The mean age at first oviposition is youngest in population 4 of the animals fed yeast or those fed yeast and *Elodea*. The number of eggs per brood, apart from density 1, is not significantly affected by density, except on a yeast diet, until 6 or more amphipods are present. However, in the present state of our knowledge these indications cannot be taken too seriously.

SURVIVAL

General.—In order to obtain a basis for statistical analysis of survival, the time taken to reach 50 per cent survival was calculated for each population density of the eight experiments. This was converted to a percentage of the total time used by all densities of each experiment. On this percentage basis densities 1 and 2 were compared with densities 10 and 20; the latter were found to survive significantly longer ($P = 0.001$). Likewise, densities 10 through 100 were significantly more resistant than isolated animals and those of lower densities ($P = 0.005$).

The above generalization indicates the greater ability to survive of the individuals living in the higher densities. This generalization is substantiated in the results of the separate experiments which are presented in survivorship curves (Fig. 5). In these curves time—that is, age in days—is plotted on the abscissae and the number living on the ordinates. The latter figure is a percentage, since the number has been corrected to an initial seeding of 100 animals. In order to avoid confusion by the interlacing of curves only 5 population densities are given for each experiment. In *Elodea* II and III only 4 population densities were used (Table 1); therefore, those experiments are presented completely.

Populations fed upon Elodea.—Three facts were indicated by the first experiment (Fig. 5, *Elodea* I), which were supported by later observations. First, the animals in a very high density (50) survived best early in life, but, as they grew older or increased in size, maximal survival appeared and was maintained in an intermediate density (25). Secondly, populations did not always follow the general trend (density 5). Lastly, the decrease in numbers living was great at first among the animals of low densities and only later in the high densities; compare the curve of the paired animals and that of the density of 50.

Elodea II and III were alike in all particulars except that in *Elodea* III the water was never changed but merely passed through silk bolting cloth weekly. Both experiments were conducted at the same time. As in *Elodea* I, maximal survival shifted in *Elodea* II from a density of 50, and, since density 20 was out of line, density 10 survived maximally from the thirty-sixth day until the end of the counts on the fiftieth day. In *Elodea* III the animals of population 8 lived best from the beginning until the end. The greater death rate, in this experiment, of populations 20 and 50 was apparently correlated with the heavily conditioned, unchanged water. This result is atypical compared to those of the other experiments.

Populations fed upon yeast.—The type of food appeared to have no effect on the influence of density upon survival. In Yeast I (Fig. 5), as in *Elodea* I and II, a dense population (100) survived maximally at first, until the nineteenth day of age. Thereafter, density 20 best maintained its numbers until the end of the counts (32 days). After its initial maximal survival, the death rate in population 100 was so great that only 42 per cent were alive at the end, as compared to 56 per cent of the single animals or 63 per cent of density 50 (not graphed). In line with earlier results the deaths were initially most numerous among the isolated animals, after that the survivors maintained their numbers.

In Yeast II and III the death rate through all populations was very high for some unknown reason. However, in Yeast II, the maximal survival of an intermediate density (10) was demonstrated again, and also the minimal resistance of the isolated animals for at least the first month of age. Yeast III provided the only exception to the observation that isolated animals never survived maximally. In fact, so irregular was the survival of the populations that it was difficult to see a trend had it not been for preceding and fol-

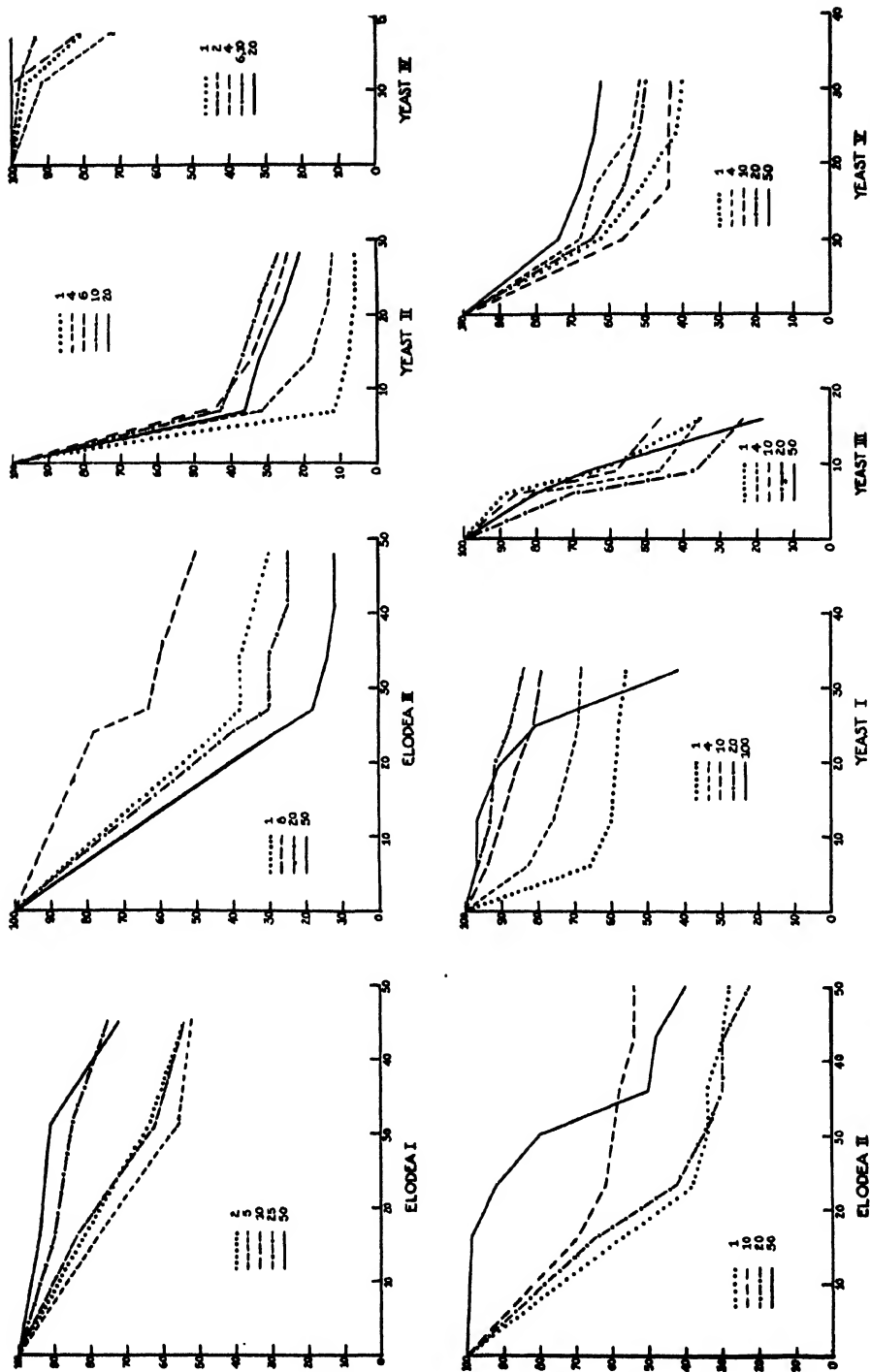


FIG. 5.—Percentage survival in the eight experiments. Ordinates, percentage alive; abscissae, time in days of age; inset key, population density plotted according to given symbols.

lowing experiments. Four days after the experiment had started, the deaths had been so numerous that replacement of animals had been made from the same stock of young originally used. In no other experiment had this been done; the results obtained may be influenced by that method.

In Yeast IV and V the highest density used—20 and 50, respectively—survived the best from beginning to end (Fig. 5), and no shift to a lower density occurred. Because of the use of chloretone, the measure of survival was restricted to 2 weeks and 1 month, respectively, in the two experiments.

In conclusion of this discussion of the effects of density upon the survival of the populations, it may be said that, although survival varied greatly from experiment to experiment and within experiments, nevertheless, a certain pattern in relation to density has emerged in nearly all cases. The type of food and water has not affected the results to an appreciable extent. With one exception (Yeast III, 6 days of age), maximal survival occurred in densities of 10 or above at every measured age. In respect to time the pattern tended to display a shift of survival from a high density to a lower (above 6), with the greatest death rate among the isolated animals and lowest densities in the first week or so of age, after which the death rate rose rapidly in the highest densities.

ENVIRONMENTAL FACTORS IN THE AMPHIPOD POPULATIONS AND DISCUSSION OF THEIR POSSIBLE EFFECTS

Increase in population density has both augmented and decreased the magnitude of physiological response in *H. azteca*. The effect has been dependent upon the particular process considered, the intensity of crowding, and, apparently, to some extent upon the conditions of the environment apart from density.

Density is a highly complex environmental factor and brings concomitant with it Smith's "density-dependent" factors of resistance (Bodenheimer, 1938), or, as expressed by MacLagen and Dunn (1936) "autobiotic factors of control." In the artificial microcosm of the experimental bottles containing the *Hyalella* populations these include directly proportional changes of carbon dioxide tension, other excretory products, fecal matter, collision frequency, and inversely proportional changes in oxygen, food, and space. Some of these have been measured (Table 6). The chemical analyses were made on water from populations seeded with adult males fed upon 4 drops of yeast. The tests started on the sixth day after seeding and ended on the eighth. No attempt has been made to measure the food consumed, but from the original distribution both food and space per animal may be estimated. Densities 50 and 100 seemed to clean up the yeast before the end of the week between feedings; however, there was apparently an excess of food at all times for the isolated animals. The 2 inches of *Elodea* appeared to provide an excess of food for all populations. However, it is realized that a quantitative excess may be qualitatively inadequate.

In Table 6 it may be seen that the 6 measured factors of the environment show a rough proportionality to the density with the exception of pH and conductivity. It is probable that the slight change in pH would have no effect, for the range is far less than that found to be effective on other organisms. The observation that both pH and conductivity dropped very slightly from density 1 to density 4 and then rose again is probably without significance, except in the fact that the two measures support each other. If significant, no explanation is at hand, or for the sudden increase in conductivity at density 50.

The question arises how these measured differentials, ingredients of the "density-dependent environment," may affect physiological processes in arthropods and whether these effects may separately or together have acted to produce the observed results on growth, reproduction, and survival of *H. azteca*.

The retardative effects of quantitative and qualitative malnutrition are too well established to need citation. In fact, von Dehn (1937) considered brood size an indication of the adequacy of the diet fed to cladocerans. In this respect the higher fecundity of the amphipods when fed throughout life upon *Elodea* as opposed to yeast is instructive, quite apart from the question of the population density. Using different diets Anderson (1930)

TABLE 6
ENVIRONMENTAL DIFFERENTIALS DEPENDENT UPON
THE POPULATION DENSITY

Density	CO ₂ * (Cc.)	O ₂ † (Cc.)	Conduc- tivity Mhos × 10 ⁻⁴	pH	Drops of Yeast per Animal	Space per Animal (Cc.)
1	52.2	5.10	2.97	7.67	4	100
2	52.6	4.96	2.97	7.60	2	50
4	52.7	4.87	2.96	7.57	1	25
6	53.1	4.67	2.97	7.60	0.67	16.7
10	54.2	4.28	3.01	7.70	0.40	10.0
20	57.4	4.06	3.09	7.70	0.20	5.0
50	66.2	3.70	3.71	7.67	0.08	2.0
Blank 1	49.8	5.61	2.75	7.83	100 cc. well water 7 days old	
Blank 2	52.5	5.53	2.85	7.80	The same with 4 drops of yeast	
Blank 3	55.3	3.95	2.96	7.63	The same with 8 drops of yeast	

* Cubic centimeters CO₂ included that given off by animals, atmospheric, and chiefly that derived from the breakdown of all carbonates and bicarbonates in the water. For the sum of the latter two factors see Blank 1.

† Both gas analyses were made by the Van Slyke method, values are the average of two readings, on the sixth and seventh days, respectively.

found that on a poor diet isolated female *Daphnia* matured later and passed through more instars prior to the release of young. Sexual maturity occurred, he stated, at a given body size rather than at a constant instar. The irregularity of sexual maturity of the isolated control amphipods fed upon a pure yeast diet may correlate with this observation. However, it is in contradiction to results from the experimental populations in which the sexually mature females of the higher population densities (less food per animal) were smaller in size and of a lower antennal segment number than those in less crowded densities (Fig. 4). A lipid factor, resembling vitamin E, appears to be necessary for maximal reproductive expression in *Gammarus* (O'Brien and Yarnold, 1937). The role of undernutrition associated with crowding was said to account in part for the decreased fecundity of *Drosophila* since many flies disturbed one another while feeding (Pearl, 1932).

The factor of restricted space decreased fecundity of lepidopterans, but the effect was less drastic than that of undernutrition (Hofmann, 1933). Space itself is not a simple factor but involves proportional changes in other factors and cannot, therefore, be used

per se without very careful analysis, despite attempts to consider it of psychological importance.

The increase in some unstable excretory products coincident with crowding is believed to cause the production of males in cladoceran cultures. By this result a depressant action is ascribed to these substances, since artificial depression of metabolism by chemical agents or by low temperature occasioned the appearance of males under conditions otherwise suited to the production of female offspring (Banta, 1937). Carbon dioxide alone did not increase male production in Cladocera (Banta, 1937), and, on the whole, there are few data concerning its action within normal range. In the tests on water from amphipod populations no distinction was made between free and bound carbon dioxide, and it is only the former which would be effective. If pH is an indication of free carbon dioxide, there is much less differential throughout the populations than the analysis for the gas would suggest. Oxygen tension might be effective in producing a proportional metabolic rate if oxygen consumption was dependent upon oxygen tension. This is, however, rarely the case over relatively normal fluctuations of tension. The results on studies of Crustacea allow no generalization (Hyman, 1929).

On the whole, from the few examples given above it can be concluded that at least undernutrition and accumulating excretory products might be responsible for the decrease of fecundity and development at and beyond a density threshold, of which the level would be determined by the susceptibility of the particular process and physiological condition of the organism. At extreme densities one or both might increase the death rate. The rapid death rate of the 100 population at an advanced age (therefore size) could be so explained. The low survival of populations 20 and 50 in *Elodea* III (Fig. 5) when the water was never changed support this view.

No evidence has thus far been presented in explanation of the continued maximal survival and early maximal growth of animals in intermediate densities. Survival at least may be related by analogy to the greater resistance of animals of lowered metabolic rate to adverse elements in the environment in accordance with the work of Child (Fowler, 1931). Evidence is also present that massed animals or their products may ameliorate a toxic or deficient environment (Allee, 1931; 1938). Such an explanation may also be applied to the results on growth and fecundity, with the added complication that in this case the yeast diet must be partially deficient, for the animals fed upon *Elodea* grew inversely to the density, while only those fed upon yeast displayed a positive density effect. The diminution of this effect on growth with increasing age may be related to the increasing strength of the density-dependent factors of resistance as the animals become larger.

Allee (1938) presents the retardative and augmentative effects of density in two curves in order to avoid the use of the terms "harmful" and "beneficial." Magnitude of physiological response is plotted on the ordinates and numbers of animals on the abscissae. The first curve (Type A) is monophasic, showing an inverse relationship; Type B is diphasic with an initially direct relationship which changes to an inverse one. The former curve could be entirely the result of density-dependent factors of resistance which increase in direct relation to crowding. The diphasic curve could be produced by the interaction of two sets of resistances, one of which would act inversely, the other directly in proportion to the density. A toxic or inadequate medium which could be ameliorated or neutralized by the products of density might be considered a resistance inverse to density, for it would retard most strongly the isolated animals. The same products of density increasing beyond the point of neutralization would form the second set of resistances, acting direct-

ly in relation to numbers of animals present. These resistances, whether they act directly with or inversely to density, are examples of density-dependent factors. At the population density in which the sum of the two sets of resistances is least, maximal physiological response would be expressed.

SUMMARY

1. *Hyaella azteca* Saussure has been used to demonstrate the effects of population density upon growth, reproduction, and survival. All populations were contained in 100 cc. of either city water (Lake Michigan), with or without chlorine, or well water and fed upon equal amounts of *Elodea* stem or yeast. The experimental animals were grown under these conditions from a few days after release from the maternal brood pouch until at least 1 month of age. The densities used ranged from 1 to 100 amphipods.

2. When fed upon *Elodea* the animals grew, at least after the second week of age, inversely to the density of the population. The adult females were of a body length inverse to density, as were those fed upon yeast supplemented after 1 month by *Elodea*. In populations fed upon yeast, on the other hand, mean maximal body or head length occurred initially in the animals of a high density, but with increasing age (or size) the maximum shifted to lower densities. Sexually mature males of density 4 were the largest, but the mean body length of females, at the time of first oviposition, was greatest in the isolated animals.

3. On a diet including *Elodea* fecundity appeared to be little affected by density until 6 or more animals were present; beyond this density fecundity was inverse to numbers present. However, on a yeast diet the females of population density 2 were significantly more fecund than those isolated, and the females of population density 4 when fed either yeast or yeast and *Elodea* released the first brood earlier than those from more or from less-crowded populations.

4. Survival of the animals varied greatly from experiment to experiment and also in respect to density. The greater survival, however, of animals in populations of 10 or over was substantiated in all eight experiments, and was not related, in any apparent way, to either the type of water or the type of food. As in the results upon growth of yeast-fed populations, an initially maximal survival in a high density (100 or 50) tended to drop to a lower density, but never below 8, with advancing age. This shift was occasioned by a late but very rapid death rate in the highest densities. In the isolated animals or low densities there was initially a high death rate, which decreased as the animals grew older.

5. Analyses of the water containing populations of 1-50 adult males demonstrated a direct proportionality between density and carbon dioxide tension, and an inverse relation between oxygen tension, food, and space. There was little effect on pH. Conductivity changed only slightly until a density of 50 showed a disproportionately great increase.

6. The above stated relation between density and carbon dioxide, presumably other excretory products, food, and space might well be responsible for inhibition of growth and fecundity and, at very high densities, of the ability to survive. It is suggested that the accelerated growth and fecundity of yeast-fed animals and the greater resistance of all animals in an intermediate density may be related to the heightened resistance caused by lowered metabolism or to the amelioration of a toxic or inadequate environment by the coincident products of density.

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